The pharmacology of 24-hour behavioural rhythms in mice.

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THE PHARMACOLOGY

OF 24-HOUR BEHAVIOURAL RHYTHMS IN MICE

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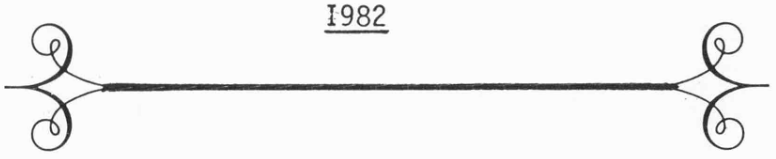

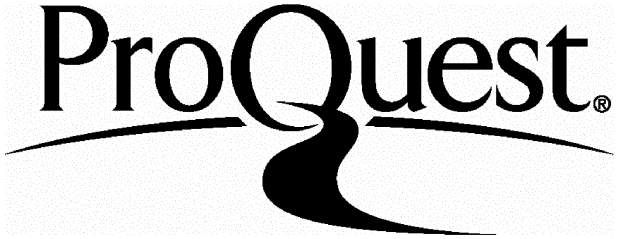

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SUMMARY

I. This study has been a broadly-based attempt to draw parallels between the known psychological disturbances in human beings following physical translocation, with behavioural changes in laboratory mice following alterations in environmental synchronizers (zietgebers).

2. The passive avoidance response was thought to provide a valid model for learning and retention. The response was found to display a significant 24-hour variation and was significantly reduced by phase shift. The reduction was alleviated by chronic benzodiazepine therapy, effects which were not found in preliminary investigations with other classes of psychoactive drugs. Benzodiazepines also exerted facilitatory effects in non-phase shifted subjects, which were maximal in the early light phase. All these properties seemed unique to the benzodiazepines.

3. Significant 24-hour variations were found to exist in a number of other behavioural parameters, namely open-field and locomotor activity, social responsiveness and aggression.

4. Levels of passive avoidance, aggression and open-field behaviour were thought to be determined by endogenous oscillators externally entrained to the environment because a) they displayed either partial or complete independence of environmental manipulations, and b) they required some period of time to re-adjust when the environmental circumstances were

changed.

5. Social and motor activity were thought to be largely exogenously controlled variations because they altered immediately in response to environmental manipulations and adjusted immediately to phase shift.

6. This study appears to confirm that "sensitive" psychological parameters can be "desynchronized" whereas the more crude motor responses appear less affected.

7. The study does not provide for the social / physical factors associated with "jet lag" in humans, and constitutes a static analogy to translocation. The study is thought to provide a useful animal model for purely desynchronization-induced psychological disturbances, and that these effects may be modified by chronic benzodiazepine therapy. Furthermore it is clear that susceptibility to this therapy may vary according to the time of administration, and allowances should be made for this.

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Chapter I

GENERAL INTRODUCTION

I.I Introduction

Life on earth is and always has been exposed to strong and rhythmic environmental influences. In considering the millions of years that living organisms have been subjected to these fluctuating conditions, it is not surprising to find that they have become conditioned to these changes or have adapted to respond to them.

In the course of evolution, a variety of organisms have developed internal (endogenous) rhythmicities whose periods match those of environmental origin. Those most commonly adopted by plants and animals are: the day-night cycle (circadian; period $\simeq$ 24 hours); the lunar cycle (circalunar; period $\simeq$ 29 days); the tidal cycle (circatidal $\simeq$ I2.4 or 24.8 hours) and the seasons (circannual; period $\simeq$ one year).

This thesis is concerned with behavioural rhythms which are adapted to the 24-hour cycle. Though referred to here as "circadian rhythms", it can later be seen that particular rhythms under study do not necessarily conform to the strict definition of the term "circadian" (later defined in this chapter). They are hereafter referred to as 24-hour rhythms.

In order to introduce the reader to the general background concerned with biological rhythms, there now follows a summary of research, both recent and historical, which broadly encompasses the animal kingdom, including man.

I.2 Historical Development

A record of biological rhythms goes back at least as far as 350 B.C. when an officer in the corps of Alexander the Great observed the "sleep rhythms" in certain leguminous plants, and Aristotle also recorded some observations of biological rhythms. The Roman orator and statesman, Cicero mentioned that the flesh of oysters varied according to the moon's cycle, while Pliny the Elder (A.D. 23-27) first attributed these observations to "lunar power".

However the phenomenon of diurnal leaf movements in plants was first scientifically investigated by the astronomer De Mairan (1729). He found these movements to continue in constant darkness, and thus postulated the existence of an endogenous component to diurnal movement in plants.

Later, Hufeland in 1797 recognized the importance of the 24-hour or solar day in functional chronology. The investigation of endogenous circadian rhythms as displayed by animal functions. however began later. In 1894, Kiesel described rhythmic colour changes in arthropods, which continued independently of light and temperature. In 1910, the Swiss naturalist, August Forel recorded the honey bee's tendency to arrive at his breakfast table at the same time each day, even in the absence of sweet material to attract them.

In the 1950s there followed a flurry of investigations into biological clocks, and the burgeoning of a new discipline which encompassed both Biology and Medicine -Chronobiology.

I.3 Characteristics of circadian rhythms

The circadian (from circa, about; dies, a day) rhythm (Halberg, I964) is probably the best known of rhythmic phenomena. It has been demonstrated in an enormous variety of organisms, from unicellular algae to higher mammals.

It is well-known to scientists that a stimulus applied at one time of day need not necessarily have the same effect at another time of day. This periodic variation has, in the past been attributed to "noise", and has been largely ignored. For example, rats when given a barbiturate may sleep for I04 minutes at one end of their night-day cycle, and for 43 minutes when an identical dose is administered at the other end of their cycle (example quoted by Cloudsley-Thompson, I980). Similarly, toxic substances may cause death to I00% of experimental animals when administered at one time of day, while at another, a 25% death rate results (see Halberg, et al, I960).

Circadian rhythmicity may persist even when all external cues (synchronizers) associated with the cycle are eliminated. These external synchronizers are also known as zietgebers (= time-giver or cue, from Aschoff, I960). For example, when isolated and placed in continuous darkness (DD) at a constant temperature, a variety of organisms including flowering plants, birds and mammals show persistence of periodicity. In its "entrained" steady state, the period of the rhythm (r) is approximately the same as that of the solar day (24 hours). In the absence of any cues (or zietgebers), the rhythm may "free run" (Pittendrigh, I960), and the natural period may vary slightly from 24 hours. Similarly, in continuous light (LL), many circadian rhythms may persist, especially those concerned with motor activity such as locomotion.

Studies such as these have revealed a generality known as "Aschoff's rule" (Aschoff, I960), which states that as the intensity of constant illumination increases, the frequency of the free-running rhythm increases for day-active species and decreases for night-active animals. Thus a manipulation which increases the frequency of an endogenous clock, relative to that of the zietgeber will cause cause the animal's oscillator to phase-lead the zietgeber by increasing amounts (e.g. this will occur in L-active animals when light intensity is increased (Aschoff, I965). These principles have confirmed in many species, but not in others (Hoffman, I965)

The characteristics of circadian rhythms may thus be summarised as follows:

(a) They fluctuate, conforming to a period of approximately 24 hours.

(b) They do not immediately re-adjust their phase following a change in environmental zietgebers.

(c) When entrained to a new period or phase, they do not immediately revert to their former period, even when the original environment is restored.

I.4 Functional significance of biological rhythms

The traditional view of biological rhythms (until the turn of the century) assigned a more or less passive role to the organism displaying the rhythm. Only within the last half-century have biological clocks within living organisms been widely recognized as conferring major survival value to those organisms possessing them. The adaptive significance of rhythms and their probable mode of evolution have been considered in several reviews (Folk, I966; Menaker, I969; Pittendrigh, I960).

Where biological clocks are found to operate, there

seems always to be some accruing biological advantage, for example, Drosophilid fruit flies emerge from pupae at a measured time at dawn (see Pittendrigh, I954), in conditions of optimal humidity. All attain sexual maturity at the same time of day, therefore maximising mating success (reviewed by Saunders, I977).

True time-sense in an animal was first conclusively demonstrated in classical experiments on honey bees by Von Frisch (I950;I954), Renner (I955), and Beier and Lindauer (I970), which showed bees to possess direction sense as well as time sense. Clearly some form of oscillatory clock allowed the bees to "anticipate" or "remember" the time of day when certain flowers presented their nectar. If confined to the hive by inclement weather, they were still able to forage successfully when they eventually emerged.

Kramer (I950) demonstrated the ability of starlings to maintain a fixed compass bearing by the sun, at the same time compensating for its movement. Since this study, a large number of migratory birds have been shown to compensate for solar and celestial movement by means of some form of internal clock. Indeed, time-compensated sun orientation has now been described in a wide variety of animals, including crustaceans, arachnids, amphibians, reptiles, insects, fish, birds and mammals (reviewed by Mathews, I973).

It is difficult to overestimate the importance of the temporal organisation of behaviour. In nature, it is vitally important that animals perform particular responses at appropriate times of the day, and seasons of the year. Thus, by achieving "steady state" entrainment to a zietgeber, an animal can partition its activities into an ordered sequence. Many animals, for example, restrict their locomotor and feeding activity to the hours of darkness (nocturnal), whereas others restrict their activities to the daylight hours

(diurnal). Still other animals are dusk-active (crepuscular). The selective advantage of this behaviour may lie in the reduction of direct competition of organisms using the same food source (reviewed by Saunders, I977).

Day-length is of particular importance, as it has been adopted by a wide variety of animals and plants as a reliable "noise-free" indicator of season. Gonadal activity and reproduction tends to coincide with environmental conditions optimal for survival of the offspring (Grocock & Clarke, I973). Daylength is also thought to be the principle means by which small mammals adjust to seasonal changes in their environment, and behaviour may be altered to anticipate critical events such as temperature changes before they occur (Lynch & Lynch, I973).

Synchrony of breeding within populations of animals is also a well-known phenomenon. As a generality, reproductive success may be improved by synchrony of birth or simultaneous emergence from the egg. For example young marine turtles are especially vulnerable to predation on hatching. However, by emerging all at the same time, they tend to "flood the predator market" and a small percentage will always survive the journey to the sea.

I.5 The Exogenous-Endogenous debate

In a natural 24-hour periodicity of light and temperature the circadian system of a plant or animal (which itself possesses an approximate 24-hour periodicity) becomes "entrained" to the environment in much the same way as two physical oscillators can achieve mutual entrainment. There is, however, some argument as to the precise role of the environment in this phenomenon. In brief, the "endogenous school" view the environment as an entraining agent in synchronizing the internal clock of an organism, and thus performing a secondary role.

They propose the biological clock to be intrinsic to the organism's physiology and to be genetically coded for, and consequently subjected to selection pressure for accuracy in time-keeping. As a result, those animals whose clocks ran fast or slow would have been eliminated.

The exogenous viewpoint, associated with Professor F.A. Brown and his co-workers, hold the environment as the "source" of periodicity in living organisms. After 30 years of study, Brown is opposed to the "inheritance" view, maintaining that subtle geophysical/ cosmic forces, which in turn are bound to daily, tidal and annual rhythms, are the master clocks which synchronize living clocks.

This dichotomy of viewpoint is, however less rigid than may be supposed. Both "schools" acknowledge the importance of endogenous and exogenous effects on the organism, and a unification of the two views may be found in Brown, Hastings & Palmer (1970).

I.6 Classification of biological rhythms

Biological rhythms may be subdivided to form three broad categories, namely:

(a) Those entirely dependent upon external influences, i.e. exogenously controlled. These rhythms are so dependent upon the environment for their functioning that removal of the external synchronizer causes disappearance of the rhythm. These rhythms tend to re-adjust immediately to temporal alterations of the environment, e.g. the human rhythm in urinary potassium excretion.

(b) Some rhythms remain impervious to environmental changes, i.e. they are endogenously controlled. E.g. the menstrual cycle.

(c) The most commonly reported rhythms are those under endogenous control, which have become entrained to an external synchronizer, but which persist with an approximate 24-hour periodicity

of their own when the controlling environmental influence is removed. Rhythms such as these are known as circadian rhythms.

I.7 Theoretical models of photoperiodic time measurement

(a) The "hourglass model": This hypothesis was put forward in I970 by C.S. Pittendrigh and co-workers. Saunders (I977) has described this model in the following terms; "a mechanism whereby an organism accumulates some end-product during one phase of its LD cycle and not the other". This type of mechanism has been confirmed in some insects (Pittendrigh, et al, I970), but does not seem applicable to mammalian time measurement (Lydick, et al, I980).

(b) "Bunning's hypothesis" (Bunning, I973): Although this model was originally proposed to account for plant photoperiodism, considerable experimental support now exists for its functioning in animals. Bunning proposed that time measurement resulted from two half -cycle components of a 24-hour rhythm, which differed in their susceptibility to light. The first I2 hours of the cycle constituted a "photophil" or light-requiring half-cycle, and the second I2 hours a "scotophil" or dark-requiring half-cycle. When light was restricted to the photophil, short-day effects resulted. Long-day effects were induced by the over-running of the photophil into the scotophil, as in the summer months. One specific form of Bunning's hypothesis involves the coincidence of internal photosensitivity with exogenous illumination and is known as the "external coincidence model". This mechanism has been demonstrated in the flesh fly, Sarcophaga argyrostoma (Saunders, I975a).

(c) The "internal coincidence model" (Pittendrigh,I960): This theory was proposed when it was noted that a change in photoperiod involved a change in the phase angle between dawn and dusk. Describing this model, Saunders (I977) ascribed only an "entrainer"

role for light, induction being a function of the internal phase relationships of constituent oscillators, i.e. two oscillators entrained respectively to dawn and dusk could serve this function. Experimental evidence in favour of this model has come from the parasitic wasp, Nasonia vitripennis (Saunders, I974).

(d)"The resonance effect" (Hamner, I963): One of the associated difficulties in the interpretation of laboratory studies is the difficulty in deducing whether light is actually inducing, or merely entraining rhythmicity in an organism (see Pittendrigh, I974). Hamner and his associates devised an experimental method which would provide evidence that observed photoperiodism was a function of a genuine circadian system. These experiments (called "T" experiments) were originally applied to plants and involved exposing an organism to a variety of different cycles, each containing a light component of the same length, whilst coupled to varying lengths of dark period. Results from "T" and "night-interruption" experiments are usually interpreted as conclusive demonstration of the circadian nature of the light-sensitive phase.

I.8 Zietgebers other than light

Light is acknowledged as the dominant time cue or zietgeber for plants and animals (reviewed by Cloudsley-Thompson, I980). Indeed light and temperature are the only environmental factors so far, unequivocally demonstrated to be coupled to the circadian clock. However Cloudsley-Thompson (I980) notes....."it is by no means improbable that some other regularly repeated stimuli, such as periodic noises, social cues or even changes in barometric pressure may also be effective phasing agents".

In our own species,social cues have been found to be sufficient to entrain circadian rhythms, even in the absence of light,

(Aschoff, et al, I97I), and experimental instructions to subjects can override illumination effects (Orth & Island, I969). Indeed in social isolation some human rhythms may free-run even in the presence of an adequate LD cycle, to which other animals would readily entrain (Aschoff, et al, I97I).

The importance of social stimuli in the synchronization of human rhythms is thought by some (e.g. Rusak & Zucker, I975) to reflect the considerable degree of control exercised by humans over their environment. As a consequence of this the biological significance of the illumination cycle has become reduced. Rusak and Zucker (I975) stress the importance of exercising caution when drawing conclusions from animal subjects, as animals are rarely tested in appropriate social situations, and the effectiveness of social cues in entraining their rhythms is largely unknown. The likely efficacy of social stimuli is well indicated by the observation that non-dominant rats in a semi-natural environment, will modify their eating and activity patterns to avoid conflict with dominant animals (Calhoun, I962). Similarly, studies on "alley cats" in New York, have shown these animals to adopt a "routine" in their scavenging movements to avoid conflict with other cats at other times of day (R.D. Passingham, personal communication).

The changes observed in animal activity rhythms after human interference with their environment (Kavanau, I969) further suggests non-light factors to play an important role in modifying mammalian rhythms. It seems that when deprived of a dominant zietgeber such as light, a rhythm may entrain to another weaker zietgeber. For example, activity rhythms in sparrows deprived of light, will "cue in" to recorded bird-song as a zietgeber (Menaker & Eskin, I966). Other putative "weak zietgebers" include electrical fields (Wever, I97I) and

cyclic pressure change (Hayden & Lindberg, I969). Some stimuli may synchronize rhythms without necessarily acting as zietgebers in the classical sense, for example varying the time of presentation of food or water will rephase the rhythm for locomotor activity in rats (Bolles & Stokes, I965), while this action can further affect rhythms in corticosterone secretion (Johnson & Levine, I973), sleep (Mouret & Bobillier, I97I) and metabolic cycles (Bobillier & Mouret, I97I).

Restricted daily feeding schedules generally result in the partial or complete synchronization of a wide range of rhythmic biological functions in rodents, resulting in pattern-changes even in such activities as conditioned lever-pressing (Boulos & Terman, I979). The latter authors suggested such a mechanism could....."enable an animal to recognise or take advantage of, the periodic recurrences of significant events in its environment". Handling of animals at the same time each day has been found to induce gonadal degeneration and restricted growth, depending upon when the handling occurred relative to the animals' LD cycle (Meier, et al, I973)

The neural mechanisms by which environmental light may gain access to the internal clock will later be outlined. However, there remains little published research on the mechanism by which stimuli other than light may synchronize internal clocks. Phylogenetic differences in susceptibility to zietgebers other than light are apparent. For example temperature cycles generally are effective as zietgebers for poikilotherms, whilst being ineffective in synchronizing mammalian rhythms, which generally show excellent temperature-compensation in preserving periodicity.

I.9 Drug-effects and chemical control of biological clocks

Hoagland (I933) originally postulated that judgement of time was mediated by chemical reactions, proceeding at a fixed rate

within the central nervous system. There now exists much supportive evidence for the chemical control and pharmacological manipulation of some biological clocks, although pharmacological modification does not necessarily imply that the clock itself has been affected.

In recent years, it has been shown that generally the period (r) of many biological clocks is remarkably intractable to a wide range of pharmacological agents, including protein synthesis inhibitors, anaesthetics and hallucinogens (see Davies, et al, I973a; Njus, et al, I974; Pittendrigh, et al, I973; Richter, I965). The few drugs known to affect r do so within very narrow limits, and these successes have not led to a clearer understanding of the chemical nature of biological clocks.

Among the drugs which have been found to affect rhythmicity are ethyl alcohol, which lengthens the tidal rhythm of the isopod Exirolana chiltoni (Enright, I97I). Small but reliable lengthening of r has also been induced in blind hamsters when these animals chronically ingested 20% alcohol (reviewed in Saunders, I977). Deuterium oxide (D_2O) or heavy water has caused consistent changes in r of many free-running rhythms. Its general effect is to lengthen r in plants and in a variety of unicellular and multicellular animals (Pittendrigh, et al, I973). For example r of the locomotor activity rhythm in the mouse increases in proportion to the concentration of D_2O in the drinking water. In a LD cycle , phase lags of entrainment are frequently recorded, and in some cases, the activity rhythm breaks away from the illumination cycle and free-runs (Dowse & Palmer, I972). Pittendrigh, et al, (I973) attributed the effects of D_2O to the diminution of cell temperature, providing supportive evidence for this, from their work with the Drosophila emergence rhythm.

Much recent research has centered on Lithium, which has been found to slow the circadian activity rhythm of the rat (Kripke & Wyborney, I980) and to cause disruption and period lengthening of the activity rhythms of goldfish (Kavaliers, I98I). Interestingly, this substance is also known to normalize some rhythms in manic-depressive illness (Tupin, I970).

The degree to which rhythms in brain chemistry affect the amplitude of behavioural rhythms remains to be established. Discrete portions of the mammalian nervous system, and the brain as a whole display circadian fluctuations in 5-Hydroxytryptamine (Scheving, et al, I968; Ancill, et al. I970), noradrenaline and acetylcholine (Saito, I97I) and dopamine (Navaratnam, I973). The amplitude of these rhythms and their phase relationship with the illumination cycle appear to be species-specific (Hery, et al, I973; Morgan, et al, I973).

Depletion of 5-HT, induced by injection of parachlorophenylalanine temporarily reduces the nocturnal feeding, drinking and activity rhythms of rats. The amplitude of these rhythms recovers to control levels within one week (Borbely, et al, I973; Fibiger & Campbell, I97I; Zucker, I97I). Similar effects have been noted with noradrenaline depletors, which may markedly reduce circadian activity rhythm amplitude, following two or three weeks of treatment (Hery, et al, I973), and may normalise three to four weeks later (Sorensen & Ellison, I973). The effects of these drugs on the free-running periods of these behaviours was not reported.

There is some evidence that noradrenergic mechanisms may link endogenous rhythms with the environmental LD cycle. For example the β-adrenergic blocker propranolol has been shown to prevent the dark-triggered increase in pineal enzyme activity in the

rat (Deguchi & Axelrod, I972). NA depletion has been further found to interfere with the entrainment of body temperature in monkeys (Winget, et al, I973). Biological time of day also appears to constitute an important determinant of the effects of NA precursors on food uptake. For example, when I-NA is applied directly to the lateral hypothalamus of rats during the L phase of a I2:I2 LD cycle, increased food uptake takes place. However when administered in the D phase food consumption is depressed (Margules, et al, I972). This effect has been attributed to the inter-relationship between NA and circadian fluctuations in the activity and sensitivity of lateral hypothalamic neurons (Schmitt, I973).

Finally, mention should be made of the effects of amphetamine and barbiturate treatment on circadian rhythms. For example, the 24-hour periodicity of eating and drinking behaviour was found to be abolished when amphetamine was added to the water supply (Borbely, I966). In a similar study, rats ingesting 20mg/kg amphetamine increased their wheel-running activity throughout the 24-hour period, with the apparent disappearance of entrainment to the LD cycle (Davies, et al, I973a). The general hyperactivity-inducing effect of amphetamine has also been found to obscure the free-running rhythm of rats maintained in constant light. The aforementioned authors have concluded that amphetamine causes disruption of the circadian activity rhythm. Furthermore, the dose-response curve for increasing activity with amphetamine has been found to be steeper in the L than the D phase of the illumination cycle (Evans, et al, I973).

The length of time spent sleeping in rats maintained on a LD I2:I2 cycle has been found to vary as a function of the time of barbiturate administration (Reinberg & Halberg, I97I), and it seems generally that time-of-day effects may constitute an important

variable in barbiturate treatment. Findings such as these suggest the importance of controlling or manipulating biological time of day, as a variable in psychopharmacological research.

I.IO Biological rhythms at the molecular & thermodynamic level

The possibility of the intra-cellular location of the biological clock has formed the basis for much recent investigation, using a variety of organisms. It now seems that nuclear RNA may play a role in rhythmicity, because of rhythmicity persistence in cells in vitro. However rhythmicity has been found to persist even after removal of the nucleus in the marine alga Acetabularia (Schweiger, I97I). Though apparently inessential for the maintainence of rhythmicity in individual cells, nuclear transplantation experiments have demonstrated the nucleus to be responsible for phase control (Schweiger & Schweiger, I965).

Experiments employing a wide range of biochemical inhibitors on the dinoflagellate Gonyaulax polyedra (Hastings & Keynon, I965), have shown that rhythmicity persisted independently of protein synthesis at the translation level, the most interesting effects being noted with actinomycin-D. Clear demonstrations of an inhibitor actually modifying a biological clock, however, came from studies on Euglena gracilis treated with cyclohexamide (an inhibitor of protein synthesis at the ribosomal level), where a concentration-dependent lengthening of free-running period was found (Feldman, I967) Clearly therefore, daily transcription from nuclear DNA does not seem necessary for the maintenance of rhythmicity.

Although there remains some doubt as to the role (if any) of translation and transcription, theoretical models for circadian rhythmicity based on such events have been formulated. Among the best-known of these theories is that termed the "chronon

concept", proposed in I967 by Ehret and Trucco. This theory suggests the genome of eukaryotic cells to contain long "polycistrons" (i.e. complexes of DNA defined as chronons), which required about 24 hours to complete their transcription. This sequential process has been likened to the functioning of a clock escapement, with a recycling mechanism enabling repetition of the process. This scheme clearly places the biological clock at the level of RNA transcription, with the periodic synthesis of enzymes responsible for rhythmic behaviour. Experimental support for this theory comes from the circadian rhythm in bioluminescence in Gonyaulax (Sweeney, I969).

Though still receiving much support today, critics of the theory point to the general lack of strong supportive evidence, and the fact that the phenomenon of temperature-independence is not consistent with enzyme function (Saunders, I977). Hastings (I970) in his review of molecular theories asks ....."what is it that oscillates.....is it enzyme quantity, enzyme location or enzyme activity?"....." it is highly likely that even if the clock does involve circadian production of RNA, there exists a large background of non-circadian production of RNA as well".

The laws of thermo-dynamics predict that an equilibrium state is defined by a condition of maximum entropy and minimum free energy, when applied to a closed system (see Sollberger, I965). The living organism however is an open system with matter and energy flowing across its boundaries. As open systems therefore, living organisms can occupy states considerably displaced from thermodynamic equilibrium, thus allowing continuous rhythmic or oscillatory processes to take place.

It is generally assumed that such oscillations arise from the operation of a control circuit operating on the principle of

negative feedback. It is well-known that negative feedback control can readily result in oscillations within a system, and experimental evidence for this comes for example from the glycolytic oscillations in yeast (Chance, et al, I964; Hess, I979). At a very general level any form of"motion"cannot proceed in one direction for ever (with the possible exception of evolutionary processes) and must at some time return to its starting point. Oatley and Goodwin (I97I) predicted oscillations to result whenever temporal constraints were applied to a system.

Taking another view, Pittendrigh (I966) suggested the original functional significance of circadian rhythms was to counteract the adverse effects of ultra-violet or visible light on essential molecular constituents of the cell. Consequently the reading and replication of the genetic message in eukaryotic cells became routinely executed in the dark.

It remains unclear however, how the accuracy of 24-hour rhythms has been achieved. Winfree (I967) has pointed out that a system of weakly interacting non-linear oscillators should be ideal "material" for events for which fixed time relationships are required. Such oscillators will readily entrain to one another, at the same frequency, as well as at higher or lower harmonics (Van der Pol, I940; Pavlidis, I969).

I.II Temperature compensation

Many papers concerning a wide variety of rhythmic phenomena, have demonstrated the general applicability of temperature independence in circadian rhythms. Examples range from unicellular algae and fungi to flowering plants,insects and vertebrates. While most the majority of physiological processes show a temperature coefficient (Q_{10}) of between 2 and 3, the period of a circadian

oscillator retains approximately the same value (i.e. a Q_{10} close to I) within a wide range of temperatures. Pittendrigh (I954) originally focussed attention on this phenomenon, noting the remarkably low Q_{10} of the free-running period of the eclosion rhythm of the fruit fly, Drosophila pseudoobscura, pointing out that an oscillator which ran faster as the temperature rose, would be almost useless as a clock.

Even in a state of induced cold torpor, diurnal rhythms of both activity and body temperature in rodents (Rawson, I960) and bats (Menaker, I959), persisted with a periodicity close to 24 hours. The available evidence from mammals thus suggests a temperature-compensated endogenous oscillator period. Furthermore, Saunders (I977) suggests this feature to be older in an evolutionary sense, than the mammalian homeostatic temperature regulation.

I.I2 Physiological bases of biological rhythms

I.I2.I non-mammalian: Although circadian periodicity occurs in plants and protozoa lacking nervous systems, the generation of rhythms in higher animals is thought to be accomplished via nervous mechanisms. Research into non-vertabrate species has however centered upon hormonal correlates of biological rhythms. Harker (I960) in a well-known study, found participation of an endocrine system in the locomotor activity rhythm of cockroaches. The source of the rhythm was traced to a cluster of neuro-secretory cells in the suboesophagal ganglion. When two animals were joined "siamese style", one was found to have adopted the rhythm of its partner. Harker's experimental designs have since been vigorously challenged (Roberts, I962), though later receiving some qualified support (Cymborowski & Brady, I972).

The available evidence from fruit flies suggests the pacemaker for pupal eclosion to be situated within the head (Zimmerman & Ives, I97I). Removal of the brain from pupae of the giant silk-worm

moth (Truman & Riddiford, I970) resulted in random or arhythmic eclosion. Subsequent reimplantation of the excised brains into the abdomens of the de-cerebrated pupae restored rhythmicity.

Brady (I974) has suggested the probable pacemaker in crustacea to lie within the inner layers of the eyestalk ganglion and to be electically coupled to the effector organs (the legs). Hormones, he said, performed a secondary role in that they affected the "level" of activity rather than its periodicity.

Apparently strong evidence exists for the non-involvement of chemical synapses and that oscillators in Aplysia are electrically coupled (Jacklet & Geronimo, I97I; Jacklet, I973). Circadian rhythms of neural activity have been demonstrated in Aplysia in a single neuron of the parietovisceral ganglion (Strumwasser, I974) and in the excised eye (Jacklet, I973).

I.I2.2 Avian rhythms: Less substantial progress appears to have been made in bird photoperiodism. However the existence of extra-retinal receptors, which were able to perceive light was confirmed by Menaker & Keatts (I968), who concluded possible photo-receptors within the brain to be coupled via the clock, to neuro-endocrine centres controlling the annual reproductive cycles. Later experiments demonstrated the eyes of house sparrows to play no part in photoperiodism (Menaker, I97I).

The avian pineal gland is thought to be the source of periodic fluctuations in activity and body temperature (Menaker, I974). It is thought by some to be a master oscillator, hierarchically entraining other oscillators, each of which is responsible for circadian rhythmicity in a particular function (Menaker, I974). Alternatively the pineal may be a coupling device between a master driving oscillator located elsewhere, and other light-sensitive

oscillators which in turn drive overt circadian rhythms.

I.I2.3 Mammalian rhythms: The existence of, and functional significance of extra-ocular receptors in non-mammalian vertebrates is well-established. However it remains most likely that ocular receptors are the principal mediators of light entrainment in mammals, although specific retinal elements involved in entrainment are unknown.

Photic stimulation has been demonstrated to evoke little response in the optic tract and lateral geniculate nucleus of albino rats (O'Steen & Anderson, I97I). However these animals could entrain their corticosterone rhythms to the LD cycle (Dunn, et al, I972), and show show light-dependent pineal respiration (Reiter & Klein, I97I). Reiter has speculated cellular elements other than classical photoreceptors transmitted to the hypothalamus, and that a humoral agent, secreted by the eye could also be involved.

However Caley, et al, (I972) demonstrated abnormal circadian rhythms in feeding by mice devoid of classical photo-receptors. These "rodless" animals also demonstrated a free-running rhythm relative to day and night. Thus rod-mediation may be important in the entrainment of feeding, by the illumination cycle.

More recent evidence from rabbits suggests the possible existence of a retino-hypothalamic feedback loop (Bobbert, et al, I979), by means of which the responses to photic stimuli are modulated according to the time-course of the light-dark alternations. The feedback loop may thus play a role in the entrainment of the internal clock in the rabbit. There is no firm evidence that pathways other than the retino-hypothalamic tract are essential for the entrainment of mammalian circadian rhythms, photic entrainment being a possible result of indirect connections between the primary optic tract and the

supra-chiasmatic nucleus (Swanson, et al, I974).

Numerous experiments have shown that discrete organs, when removed from the organism, are capable of periodicity of such actions as secretion and contraction. The adrenal gland (Andrews, I969) continues rhythmicity in secretory activity in complete isolation from the organism and from neural influences. Similarly, the small intestine may continue rhythmicity of contractions in vitro (Bunning, I973). In the intact organism, however, the phasing of these rhythms is subject to control by central neural pacemakers.

I.I2.4 Human rhythms: The importance of the hypothalamus in the regulation of rhythmicity in humans is supported by much clinical data. For example patients with hypothalamic damage show disruption of rhythmicity in the normal pattern of electrolyte excretion (Krieger & Krieger, I967). Indeed rhythmic changes of electrical activity have been found to occur in the lateral hypothalamus (Schmitt, I973), and the adjacent medial preoptic region (Johnson, et al, I97I).

Physiological studies of humans isolated from environmental time cues have demonstrated the presence of at least two major pacemakers in the human circadian system. One apparently drives the sleep-wake cycle and the other controls the rhythm for body temperature (Czeisler, et al, I980). These rhythms may sometimes desynchronize and run independently of one another.

The supra-chiasmatic nucleus (SCN), within the hypothalamus (the control centre of the autonomic nervous system), has now been established as a probable source of rhythmicity in humans. This assertion corresponds to findings in primates (Moore, I979; Lydick & Moore-Ede, I980) and in lower animals (reviewed by Rusak & Zucker, I979). Histological demonstration of the SCN in man has

recently been published (Lydick, et al, I980) while structure and function of the SCN in the squirrel monkey have been further elucidated (Lydick & Moore-Ede, I980; Moore-Ede, et al, I980 respectively).

Lydick, et al (I980) have reported the SCN of the human brain to be homologous to the non-human primate, though more diffusely organised and laterally placed than in the latter.

I.I3 The putative role of cell-membranes

Recently, much interest has centered on the control of rhythmic biological processes, as a function of the stability/ instability of cell-membranes. One possible mechanism of drug action on rhythm period, is that they influence the permeability of specific ionic channels (Eskin, I974; Njus, et al, I974; Strumwasser, I974). Substances such as D_2O and alcohol which lengthen the periods of various clocks, have all been described as increasing the stability of biological membranes (reviewed by Saunders, I977).

One model of the circadian clock (Njus, et al, I974) suggests that timing is the result of a feedback system involving membrane ion gradients and protein-dependent ion transport channels. This model accounts for temperature compensation of rhythm period, on the basis of the temperature adaptation of the membrane lipid bi-layer. The rate of ion diffusion and of the horizontal movement of proteins responsible for ion transport, would both be compensated for temperature change by this mechanism. The fact that K^+ ions (Bunning, I973) can produce phase-shifts similar to those induced by photoperiod change, suggests a crucial role for K^+ in the production of circadian rhythmicity, and its entrainment by light.

I.I4 Human circadian rhythms

As in numerous plant and animal species, the circadian

cycle is firmly established as a vital feature in man. Diurnal rhythms in physiological variables were first described for pulse-rate (Autenreith, I80I), gaseous exchange during respiration (Prout, I8I3) and evaporation (Reil, I823). There also exists undisputed evidence for periodicity of human birth (Charles, I853).

It has become increasingly clear that many rhythmic physiological functions persist regardless of the state of rest/activity of the subject. For example, Colquhuon (I97I) has demonstrated a diurnal variation of body temperature, the value rising suddenly on waking, gradually reaching a peak at I800 hours, then dropping rapidly after 2I00 hours with the trough at around 0300-0400 hours.

Numerous other psychological and biochemical variables such as blood pressure, urine and blood constituents, 5-Hydroxy-tryptamine concentration in the blood and even human lactation, show a 24-hour periodicity and have been reviewed in detail by Mills (I966) and Mills and Conroy (I970). A majority of these physiological rhythms are truly endogenous, and have been verified in isolated conditions in caves (Kleitman, I963) and the constant illumination periods provided by the arctic (Lobban, I960)

I.I5 Mental disorders

With a gradual increase of knowledge of circadian rhythms, their clinical implication is becoming clearer, especially in relation to diagnoses of various illnesses. Disturbances in the circadian rhythmicity of biological function have been reported in various mental disorders. Spontaneous alterations in the normal patterns of the sleep-wake cycle and daily variations of mood, are frequently associated with psychiatric illnesses (Conroy & Hall, I968). Stevens, et al, (I959) demonstrated a positive correlation

between mood and 24-hour excretion of 5-Hydroxyindole acetic acid. Sarai and Kayano (I968) have shown a correlation between depression and serum 5-HT levels.

Monroe (I967) found "poor sleepers" to be individuals whose physiological rhythms were not adjusted in synchrony with the local 24-hour clock. Cahn, et al, (I968) in their study of normal volunteers in isolation, suggested that desynchronization among internal rhythms may be associated with depression. Indeed asynchrony and an inability to respond effectively to a phase shift is thought to characterize a pre-suicidal state (Rockwell, et al, I97I).

Bunney and Fawcett (I965) noted high levels of I7-Hydroxycorticosteroids (I7-OHCS) in hospitalized depressives, with a high correlation also found, between the severity of the affliction and the 24-hour excretion of I7-OHCS. This raised the possibility of predicting suicide attempts on the basis of altered biological parameters. Yamashita, et al (I969) have also reported disturbances of plasma I7-OHCS rhythms in acute schi zophrenics.

I.I6 Psychological function

Since biochemical processes exhibit marked circadian variation, it follows that psychological processes could also vary as a function of these underlying physiological correlates. The human circadian rhythm in work and performance has been reviewed in detail by Colquhuon (I97I). It seems that the individual's capacity for mental or physical work fluctuates throughout the waking period (e.g. Blake, I967a). However the precise nature of this variation is a matter of debate. For example, differences between functions have been attributed to individual personality differences. Blake (I967a; I97I) has shown the circadian rhythms of introverts and extraverts to differ in such parameters as peak arousal times, while Patkai (I97I) further

extends this to differentiating individuals on the basis of their being "morning" or "evening" people.

However, while this research has proved invaluable in accounting for minor inter-individual differences, it does not account for intra-individual differences over different times of the day, on performance on a variety of different tasks. Performance on these different types of task show characteristic variations over time-of-day, for example, short-term memory shows an improvement from early to mid-morning, and a fairly steady fall over the rest of the day (Hockey & Colquhuon, 1972). Similarly Baddeley, et al, (1970) found a decrease in digit summation ability, from mid-morning to mid-afternoon. This function appears to be generally applicable in many contexts, as published studies with male and female schoolchildren (Winch, 1912a;b; Gates, 1916a;b), naval ratings (Blake, 1971) and undergraduates (Gates, 1916b; Baddeley, et al, 1970) show consistency in the time-of-day effects on memory.

Kleitman (1963) has proposed that the arousal level of an individual varies, showing a tendency to increase throughout the day, and is a function of a rest-activity cycle that persists throughout the sleep and waking states. It seems also that short-term memory may be impaired under conditions of high arousal (e.g. Kleinsmith & Kaplan, 1963; Walker & Tarte, 1963; McLean, 1969), and the inverse relationship between arousal and mental efficiency has been pointed out in a number of studies (reviewed by Colquhuon, 1971).

The suggestion that arousal levels increase throughout the day is based on the general findings that both body temperature (Blake, 1967b; Colquhuon, 1971), and performance on a number of different tasks, such as cancellation, card-sorting and calculation (Blake, 1967a) show a fairly continuous rise throughout the day,

peaking at about 2000 hours. Typically, the tasks that show this continuous rise involve little, if any, memory load, and have been characterized by Alluisi and Chiles (I967) as involving "performance stress", and by Hockey and Calhoun (I972) as demanding ...."a more immediate processing of information". Thus, what these tasks have in common is that they require sustained attention, and in most cases because they are timed, involve considerable speed stress. Blake (I967a) also noted that in the self-paced tasks, it was speed rather than errors which proved sensitive to the time of day. In brief, therefore it appears that the ability to "cope" with tasks involving "performance stress" increases as the general arousal level of the brain increases, with short-term memory correspondingly decreasing.

I.I7 The phenomenon of "Jet lag"

In the past 30 years, there has been increasing interest and concern into the medical and neurological aspects of sub-sonic and super-sonic travel and its effects on the ordinary citizen (e.g. Gooddy, I97I). Many elaborate studies have been carried out for the armed services and N.A.S.A. The papers of Hauty and Adams, (I966a;b) are among the best-known attempts at analysing this problem.

Conroy and Mills (I970), referring to the work of Hauty and Adams has stated....."there is growing concern that much of the discomfort and decrease in efficiency suffered by long-distance air travellers is due to the disturbance of physiological rhythms, rather than to fatigue induced by flying". It seems that flight can cause dysrhythmia because the internal clock has to re-adjust to the new and unaccustomed environmental cues.

The problem of internal desynchronization is of particular importance to air crews and relevant to ministers and diplomats, who may affect many other lives other than their own, by

general mental inefficiency and the making of improper decisions, whilst suffering the effects of disorientation. The development of subsonic and supersonic aircraft has meant that half-a-dozen time-zones or more can be crossed in 6 hours. Thus man is exposed in an unnaturally short time, to a different day-night cycle to which his body is synchronized. Following this "phase shift" of the external synchronizers of the internal rhythm, there is inevitably some delay before the internal rhythm is able to "catch up" and re-adjust itself to the new environmental schedule. During this delay, the symptoms of "jet lag" will manifest themselves. Pittendrigh (I960) describes this delay in the following terms....."transients always precede the attainment of a new steady state.....this is true, whether the former steady state was disrupted by a single perturbation or by a phase shift in the entraining cycle".

Wever (I966) in a significant contribution to the study of desynchronization, developed a theoretical model for the re-adjustment of circadian rhythms, suggesting the duration of re-entrainment depended on;

(a) The amount (or degree) of phase shift.

(b) The direction of shift (whether advancing or delaying)

(c) The phase-angle difference between organism and zietgeber.

Since most experiments, especially those relating to time-zone transition, are concerned with 24-hour rhythms. the phase-angle difference is zero and can be disregarded. However where experiments have been conducted either in isolated or abnormal time conditions, the phase-angle can vary and must be taken into consideration. Information which led to the formulation of Wever's

model is summarised as follows:

(a) The time taken to re-entrain to a new cycle tends to be shorter after a 6-hour shortening, than after a 6-hour lengthening of the zietgeber.

(b) The re-entrainment time after phase shift in either direction is shorter after a single change of dark-time, than after a single change of light-time.

Following extended translongitudinal air travel, (4 hours or more), the abnormal phase relationship of the internal biological processes to the new environmental time cues, may elicit profound feelings of disorientation and subjective feelings of "detachment". Diplomats and company executives have reported difficulties in arriving at decisions, normally "second nature" to them under normal circumstances (Conroy, I97I). Gerritzen and co-workers (I966) reported impairment of discrete and sensitive motor and sensory tasks, when subjects were subjected to an onset of constant light. Gross motor functions however remained unaffected. The affected functions re-entrained themselves after 3 days of constant light, though they fluctuated with a period slightly longer than 24 hours. The implications of findings such as these for airline pilots are clear. Indeed, Siegel (I969), in a study of desynchronization in airline pilots, has suggested the most likely occurrence of air accidents, when the pilot's rhythm has been disrupted. Statistical information supports this suggestion, in that air accidents due to pilot error are more likely to occur at night, on take-off and landing, and after prolonged travel.

One of the most familiar problems for the air traveller is insomnia during the local hours of darkness, following arrival at his destination. The inability to establish satisfactory

sleep patterns for several days after arrival is also a well-known problem. The implications of this for military personnel have been reviewed by Nicholson (I970b), and for air crews by Nicholson (I970a; I972). It has been reported that air crews generally find the Eastward flight more strenuous than the Westward (Preston & Bateman, I970). The more acute sleep disruption and deprivation following the Eastward flight, in turn affected reaction speeds. Similarly, Halberg and Reinberg (I967) reported greater disorientation and sleep disruption on an Eastward trnslocation than a Westward flight, in subjects on a return trip between the U.S.A. and West Germany. Conroy and Mills (I970) reported that in a subject flying Westward, from Manchester to Chicago, there was considerable disruption of the sleep-wakefulness cycle. During the first 4 days after the flight, the subject felt fatigued at around I800 hours, which corresponded to midnight in Manchester. The rhythm re-adjusted to the new schedule after 6 days.

Clearly then, both East and West flight directions are disruptive. A consensus of the literature appears to indicate greater ill-effects following Eastward transmeridional flight, than in other compass directions (this corresponds to a phase advance of the 24-hour rhythm .

I.I8 Phase-shift and psychological function

The existence of circadian variations in such psychological parameters as mental efficiency and reaction time, is well-established (Colquhuon, I97I;I972), and the latter author claims these to be determined by underlying physiological rhythms. Klein, et al, (I970) examined the effects of multiple time-zone travel on pilot-efficiency, and found such performance to reveal a sinusoidal 24-hour variation, with the position of the peak and trough, between

I400 and I500 hours, and 0400 and 0500 respectively. There was disruption of the circadian variation of efficiency following 8-hour flights in both Easterly and Westerly directions. However the decrement in performance level was far greater following the Easterly translocation.

In another study (Klein, et al, I968), phase shift was found to induce the most significant ill-effects in psychomotor function, while such parameters as digit-summation ability remained comparatively unaffected. There were also further indications that the disruption in psychomotor function was more pronounced after an Eastward shift (advance), than following a Westward (delay) shift.

In considering time-zone effects, it is important to distinguish between the specific effects of altered time-schedules, as distinct from the physical act of flying. Miller (I968), and Cantrell, et al (I97I) have presented a review of the effects of flight-stress and fatigue, which is summarised as follows:

(a) Disturbances due to desynchronization are not evident following a Northerly or Southerly flight.

(b) The act of flying is not the only factor which elicits behavioural disturbances following the flight.

(c) The duration of resynchronization period is dependent upon a number of factors. Among these are (1) the degree or magnitude of time-zone shift,(2) the nature of the particular rhythm under study, (3) the direction of transition, and (4) The time at which phase shift was initiated.

Thus, although "flight stress" may play a role in augmenting phase-shift-induced disturbance, clearly one requires more than the physical process of flying to account for these disturbances.

Among the characteristic effects of internal

desynchronization, one of the most notable is the significant depression of the 24-hour mean values for many parameters, immediately after flight. Klein, et al (I972) concluded that the depression of the mean was related to the time-in-transit, rather than to the number of time-zones crossed. These authors also found a more pronounced depression following an Eastward translocation, especially after a night flight. Diminution of the amplitude of a circadian rhythm is a common trend seen in the body's adjustment to a changed relationship between its internal clock, and the external time-cues. This phenomenon has also been observed in shift-workers (Browne, I96I).

Cantrell, et al (I97I) have reviewed the relative resynchronization periods of various parameters, and have concluded that the more simple psychological measurements such as reaction-time tend to resynchronize more rapidly than the more complex performance tests.

I.I9 The importance of direction of phase shift

As has been previously mentioned, though Eastward translocation seems generally more disruptive in its effects, contradictory results have been obtained, concerning the relative re-adaptation periods following both East and Westerly flight. For example, Mohler, et al (I968), and Siegel, et al (I969) found a shorter re-adaptation period following an Eastward flight, than a Westward flight. Some further experimental support for this has come from studies employing both humans and birds (finches) (Aschoff, I969)

Results such as these tend to corroborate Wever's (I966) mathematical model which predicted marginally quicker re-adaptation following an Eastward flight. These results sharply conflict with those of numerous workers such as Hauty and Adams

(I966a;b), Hallberg & Reinberg,(I967), Halberg, et al, (I967) Wegmann, et al (I970), Klein, et al (I970; I972) and Elliott, et al, (I97I). These workers in general, found greater disruption of the human circadian system following a phase advance, constituted by an Eastward translocation.

Aschoff (I969) has postulated that gradual adaptation to a Westward flight would involve a series of cycles rather longer than 24 hours, which could be accomodated by the various oscillators rather more easily than a series of shortened cycles, as in Eastward flight. This is corroborated by the finding that human subjects in isolation adopt a cycle longer than 24 hours.(Aschoff, I969). It does seem that in the majority of cases, desynchronization symptoms were more marked, and resynchronization duration longer following a phase advance due to an Eastward flight.

Investigations on work-rest schedules (Preston & Bateman, I970) have shown the ease of extinction of the old rhythm to be greater when a subject can immediately commence a new sleep cycle, as would be the case in a Westward flight. In fact pilots on round-the-world schedules seem to prefer Westbound flights, maintaining that "they find a 29 hour day, i.e. waiting for local time to catch up, less tiring (Preston & Bateman, I970). The depth of sleep from which one is aroused has further been shown to directly influence performance. This has led Klein, et al (I972) to postulate that after translocation, the impairment of performance as a function of particular times of day is a consequence of a persisting rhythm of sleep/wakefullness, with a displacement of light and deep sleep periods.

The information available on the effects of rapid time-transition on bodily functions is still scanty, but is sufficiently

adequate to indicate that the physiological and psychological effects of such journeys are considerably longer-lasting, and of greater consequence than previously appreciated.

I.20 Aims of this thesis

The rationale for this investigation is to study the importance of time in the control of behaviour, both overt and otherwise, together with the effects of temporal changes of the environmental controlling influence on behavioural and psychological function. The ways in which these effects can be pharmacologically modified will form an important component of the study. Analyses of the underlying physiological / biochemical processes are not attempted, though speculative comments are made. The importance of the environment in controlling or entraining behaviour is also of particular interest.

A simple conditioned avoidance task (the passive avoidance response) for laboratory mice is used as a simple animal model to demonstrate the existence of time-of-day effects in learning and memory. Broadly speaking this thesis is subdivided, dealing with passive avoidance on the one hand, and further behavioural parameters on the other. These behavioural parameters have been selected on the basis of their possible relevance in the assessment of emotion and anxiety states. Time-of-day variables, together with the effect on these responses, of simulated time-zone transition is examined, by employing phase shifts in the animals' illumination cycle.

The effects of drugs classified as "minor tranquillizers", together with some other clinically-important, centrally-acting drugs is examined. Their time-of-day action on behaviour, and their inter-relationships with phase shift, will it is hoped, provide information as to possible beneficial and

therapeutic possibilities. The effects may possibly be paralleled in humans and consequently provide some practical clinical usage.

Chapter 2

MATERIALS AND METHODS

2.I Subjects

Experimental animals were albino male CFLP mice, from an outbred strain supplied by the animal facility, School of Pharmacy and Pharmacology, the University of Bath. Unless otherwise indicated, these animals were subjected to a pre-experimental illumination cycle of LD I2:I2, in an animal-holding room adjacent to the experimental laboratory, under controlled conditions of temperature. Pre-experimental animals were housed in standard poythene, wire-topped cages, of measurements 50x32xI8cm., containing sawdust. Cages were located in a special rack which included a constant watering device. On commencement of each experiment, a cage was placed inside an environmental cabinet, later described (see fig. I).

Handling and extraneous noise in the holding room were minimised as such stressors have been shown to influence adreno-cortical function (Bowden, I979). Care was taken to ensure that all experimental mice were as "standardised" as possible. Parameters such as age, sexual experience and duration of prior housing have all been shown to influence endocrine response, and may consequently change the behavioural characteristics of the animals (Brain & Nowell, I97I). For reasons later discussed, all experimental animals were tested at 7 weeks of age.

FIGURE I (overleaf)

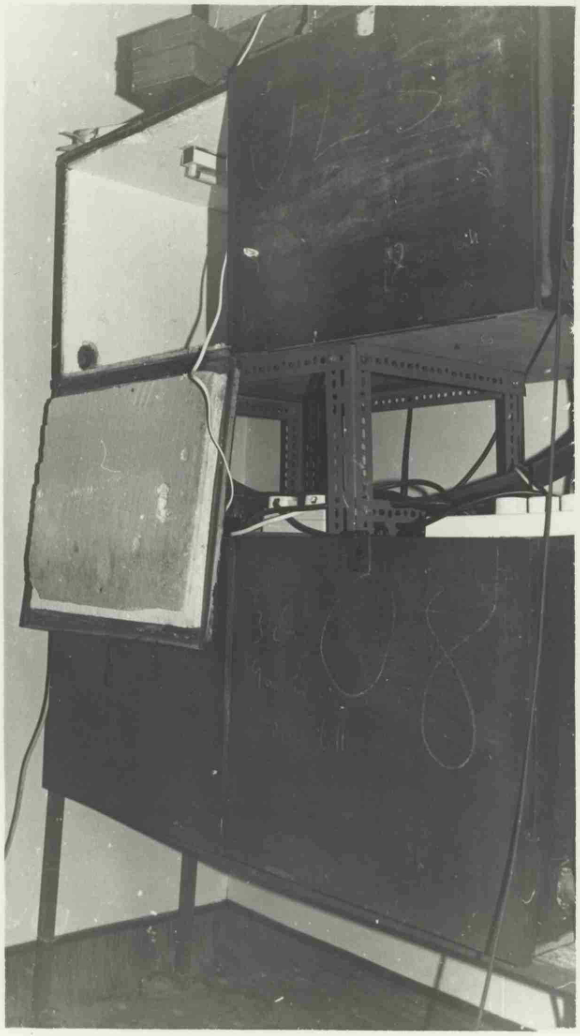
ENVIRONMENTAL CONTROL, RECORDING & CONDITIONED AVOIDANCE APPARATUS

TOP LEFT; Environmental control cabinet.

TOP RIGHT; Infra-red camera. *Positioned vertically above cabinet with ventilation trunking in foreground.*

BOTTOM LEFT; Video Equipment. *Includes monitor, deck, cassette tapes, and timer.*

BOTTOM RIGHT; Passive avoidance apparatus.



2.2 Behavioural analyses and statistical evaluation

All statistical tests employed are nonparametric, methods for which are described in Siegel (1956). All experimental sample sizes were =11, and all diagrams display the mean ± standard error. 5% was taken as the lowest reliable level of significance. The tests applied were as follows:

(a) In all cases where independent samples have been compared, the 2-tailed Mann-Whitney 'U' test has been applied.

(b) Where correlations were sought, the Spearman rank correlation coefficient test was applied.

(c) Where non-random sequences of results were sought the one-sample 'runs' test was applied.

2.3 Environmental control

To circumvent the problem of continuous sampling throughout 24 hours, experimental animals were housed in "environmental cabinets", constructed from 15mm blockboard, with dimensions of 61x61x 46cm. (see fig. 1). To ensure light-proofing, 13mm plastic foam strips were sandwiched between adjoining faces. To increase sound insulation, each cabinet was lined with 13mm. polystyrene sheeting, thus excluding sound levels below 76dB at audible frequencies. The door to each cabinet was hinged along 46cm. of the bottom edge, and closed with a latch fitting.

The interior to each cabinet was illuminated by a 12" miniature, 8 Watt, warm white fluorescent light (Thorn electonics, LJ S100 8H) with the choke outside the cabinet to exclude additional heat. The light was controlled by a time-switch (Sangamo Type 5254-I-171). Further details and diagrams have been published elsewhere in Hillier, et al (1973).

The cabinets, eight in all, were maintained in the

animal holding room, where the temperature was maintained at a constant 23°C.± 2.0°C. by thermostatic control. The internal temperature of the cabinets was also maintained at a constant level, by means of a constant stream of air drawn through the boxes by a domestic extractor fan (Xpelair Ltd., Birmingham) connected via 32mm. bore rubber tubing. The fan also had the effect of removing accumulated heat and water vapour.

A constant watering device was included, with food (standard laboratory chow) obtainable ad libitum from extra-large food hoppers, integral to the wire-topped cage. Thus animals could be left undisturbed for 2 weeks.

All animals were subjected to a I2-hour light/ I2-hour dark cycle unless otherwise stated, with pre-experimental light period in the holding room, running from 0600-I800 hours local time. When disturbing the animals for feeding / cleaning purposes, care was taken to ensure the irregularity of such movements, as disturbing the animals at regular times of day could constitute an additional time cue.

Confinement of animals within a cabinet thus made possible the deployment of different lighting regimens in different cabinets, enabling convenience to the experimenter, and sampling to be carried out over a normal working day.

An acclimatization period of I6 days was always allowed when cabinet-confinement involved a phase shift of the light cycle from that of the holding room. In any event, at least I0 days were allowed for adjustment to the new environmental conditions within the cabinet.

2.4 Passive avoidance apparatus

The passive avoidance apparatus employed has previously

been described (Boissier, et al, I968) as a method of screening minor tranquillizers in mice. It consisted of a transparent, perspex box of internal dimensions 23xI8xI5cm., with a transparent lid (see fig. I). The floor was covered with four rectangular aluminium plates of IIx8cm., with adjacent plates separated by 4mm. gaps. Each plate was connected to a selector panel, supplying 0.2mA continuous current, and consisting of 4 switches, which enabled a circuit to be made to the desired plate. The apparatus was connected to the mains via a transformer,supplying variable voltage of up to 500v. When administering shock, a constant current of 0.5mA and 500v was established for 0.5 secs. at the appropriate plate.

2.5 Passive avoidance testing procedure

At the beginning of a test, a mouse was gently dropped onto a plate and allowed to explore the enclosure for I5 secs. In the ensuing 60 second period, each attempted plate-crossing was punished by a shock administered at the plate to which the crossing was attempted. In the instances where an animal attempted to "reverse" to another plate, no shock was administered.

Thus, the total number of times that the animal crossed or attempted to cross from one plate to another, during this one-minute period was recorded.

2.5.I Acquisition

The administration of shock suppressed the natural exploratory behaviour of the animal which "learned" to avoid the stimulus by remaining on one plate, i.e. it had acquired the passive avoidance response. If the animal had not learned, it continued to cross from plate to plate, irrespective of shock, thus registering a higher score with respect to those animals which had acquired the response.

FIGURE 2 *(Overleaf)*

ACTIVITY-RECORDING EQUIPMENT

TOP; Standard polythene cage. *Within the environmental cabinet, underlaid with "Varimex" sensor.*

CENTRE; "Varimex" activity-recorder.

BOTTOM; The open field

FIGURE 3 *(Overleaf)*

SOME SOCIAL BEHAVIOURS OF LABORATORY MICE *(see Grant & Mackintosh, 1966)*

TOP; Social investigation:

Mutual sniffing and other activities directed towards a test partner.

CENTRE; Groom:

Mouthing and licking of the fur of a test partner. In isolated and socially-deprived animals, this activity frequently escalates to a more intensive form, followed by biting.

BOTTOM; Offensive sideways posture:

A ritualized manoevre exhibited by the attacking animal (right). Note slitted eyes and flattened ears compared to the submissive animal (left), which pushes away with forepaws.

2.5.2 Retention

Following this initial exposure to the apparatus, each animal was again subjected to a passive avoidance trial, exactly 24 hours later, employing an identical procedure to that adopted in the first trial. This time the number of shocks administered was deemed to reflect "retention" of the response from the previous day, i.e. the degree to which the subject had "remembered" the previously-conditioned response. The degree of retention was found by calculating the percentage difference between first and second exposure scores, by the formula:

$$\frac{\text{Ist trial score - second trial score x 100}}{\text{Ist trial score}}$$

Thus a low second trial score, reflecting a high level of retention, would be indicated by a higher percentage difference between the two scores (i.e. a high percentage "correct responses"), whereas a low percentage correct responses would be shown by a higher second exposure score, and consequent lower percentage difference. A similar method of calculating passive avoidance retention has been applied to rats (Davies et al, 1974), though employing different methods and apparatus.

2.6 Monitoring of locomotor activity

In order to transduce home-cage activity into a permanent record, cages were underlaid with a "Varimex" horizontal electromagnetic sensor (see fig. 2). Gross activity by the animals was then transposed to a digital readout on a "Selective activity" control unit (Varimex Ltd., Columbus Instruments, Columbus, Ohio; see fig. 2). Aggregate scores for a one-hour period were then obtained at two-hourly intervals.

2.6.I. Open-field activity

In order to record motor responsiveness to a novel environment, an open field apparatus of standard geometric design was deployed. The arena was divided, to form 8 inner segments, with a corresponding number of wall-segments (see fig. 2). The height of the wall was 50cm. Extraneous light was excluded by means of a lid I.5m. above the arena, and a surrounding opaque curtain. White illumination of constant intensity was provided when needed, by a 30 Watt bulb beneath the lid.

All open field trials were videotaped through an aperture in the lid, with the camera positioned vertically above the apparatus, the advantage of this method being the absence of extraneous disturbance by the experimenter.

Each trial involved placing an animal at the centre of the arena, and then recording the number of segment-crossings with a hand-counter. All foreign matter, faeces and urine etc., were removed after each trial with soap and water.

2.7 Video-recording

To facilitate behavioural observation in the animals' dark phase, an infra-red "surveillance camera" (Ikegami model ITC) was positioned vertically above an aperture in the roof of a cabinet (see fig. I). A specially-constructed light-proof box was positioned intermediate to camera and box, facilitating adequate focal length (not less than 50cm.) for the camera (fig. I). A 30 Watt bulb and infra-red filter, confined in a small box adjacent to the camera, provided an illumination source for the camera.

The design of the camera was such that compensation was made for the lights-on period, thus enabling clear observation in both light and dark phases. Recording was made by a JVC model CR

6060ET video-recorder with extended stop-frame facility, using Scotch UCA cassette tapes of one hour playing time. Recording playback was analysed on a video-monitor (Shibaden model VM I7I-K). A time-switch (Horstmann HPKY, Horstman Ltd. Bath) in series with the video-recorder enabled automatic recordings of desired duration to be made at regular intervals throughout the day and night (see fig. I)

Chapter 3

THE PASSIVE AVOIDANCE RESPONSE

3.I Introduction

It appears that definitions of "learning" vary widely and no generally accepted definition exists. It is normally taken to refer to changes in the central nervous system, culminating in the formation of a "memory trace" in the brain (for reviews, see Thorndike, I93I; Tolman, I932; Hilgard, I95I; I956; Hilgard & Marquis, I96I; Jarvik, I969). It has also been defined as... "a change in behaviour as a result of some practice or observation" (Kimble, I96I). The term "learning" in general relates to an initial period, during which a new habit is acquired, and relates to the intervening variable or underlying storage process, by which a relatively permanent change in behaviour may take place (see Squire, I976). The measurement of memory after learning has taken place, is usually considered to be a test of "retention".

Hebb (I949) evoked the term "consolidation", referring to the gradual conversion of memory from a labile "short-term" form, to a more permanent "long term" form. There remains however, considerable uncertainty as to the time-course of this hypothetical process, and its biological basis (Squire, I975).

The two behavioural models most commonly employed in the study of learning and memory are appetitive responses (e.g. reward of food, water etc.) and aversive learning (e.g. footshock). Of the latter category, the passive avoidance response is frequently used in order to measure an organism's learning capability. In order to determine whether any lasting effects have resulted from this "learning experience", a second trial may be carried out at a given time interval, to measure the organism's ability to respond appropriately, i.e. whether it "remembered" the learning experience.

The primary feature of the passive avoidance test is

that an animal learns to associate a noxious stimulus with a particular response. Following this initial experience it can avoid the noxious stimulus in the future, by changing its behaviour and avoiding the particular response which originally led to punishment. This method is commonly employed with rodents, where, for example, rats may easily be conditioned against their natural tendency to seek dark places (see Davies, et al, I974).

Mention must be made, however, of the important distinction between passive and "active" avoidance learning, where the subject is required to execute a response in order to avoid the noxious stimulus, rather than passively avoid the stimulus.

In this study, the passive avoidance response in mice has been used as an index of learning and memory. The method (outlined in chapter 2) involved the administering of electroshock at each attempt to cross from one metal plate to another. The intensity of shock was insufficient to cause a high level of pain and violent "escape reactions". It was sufficient merely to "dissuade" the animals from crossing from plate to plate. A second trial, carried out 24 hours later, determined the extent to which subjects had memorized the response.

Unless otherwise stated, the intervening period between first and second trial was always 24 hours, in consideration of the "Kamin effect" (Kamin, I957), i.e. retrial intervals of other than multiples of I2 result in retention deficits.

3.2 Preliminary investigations

The importance of retaining inter-experimental consistency in subject animals cannot be overemphasized. For this reason, a series of pilot experiments were carried out in order to determine (a) possible variables likely to adversely influence the

desired "standardization" of experimental animals, and (b) whether these parameters would be likely to result in variance between groups of animals in the same experiment. The chosen parameters were; age, body weight, social rank, illumination conditions, presence of shock, daily handling and injection.

3.3 Methods

Procedure involved an initial exposure, followed by retrial 24 hours later. All testing took place circa I200 hours (i.e local time and mid light phase), and the following experimental designs were applied:

(a) Effect of age: 2 groups of animals, aged 4 weeks (n=I2) and I0 weeks (n=I2) were compared.

(b) Effect of body weight: 60 stock animals (7 weeks of age) were constituted, from which two groups were formed, of mean weight 34g (n=I2) and 52g (n=I2).

(c) Effect of social rank: I00 stock animals (7 weeks of age) were constituted, from which 2 groups were formed, comprised of dominant (n=I4) and subordinate (n=I6) individuals. These were identified and marked with fur dye, in their home cages in the holding room. They were observed for one hour on a daily basis, for 7 days and were designated dominant if at least 5 attacks were initiated upon cagemates, without themselves submitting to an attack. Similarly an animal was designated "subordinate" if it was never seen to attack a cagemate, and itself submit to attack and vocalise on at least 5 occasions (see fig. 3). Postural criteria have been adopted from the definitive study on laboratory rodents by Grant and Mackintosh (I963). Animals were 8 weeks of age when tested.

(d) Effect of illumination: Two groups were taken from a dark environment (at mid dark phase), with one group (n=I2) tested under light and the other (n=I2) in darkness under red light. A

FIGURE 4

EFFECT OF AGE ON THE PASSIVE AVOIDANCE RESPONSE.
TESTED MIDDAY. n=12 ±S.E. $p<.02$

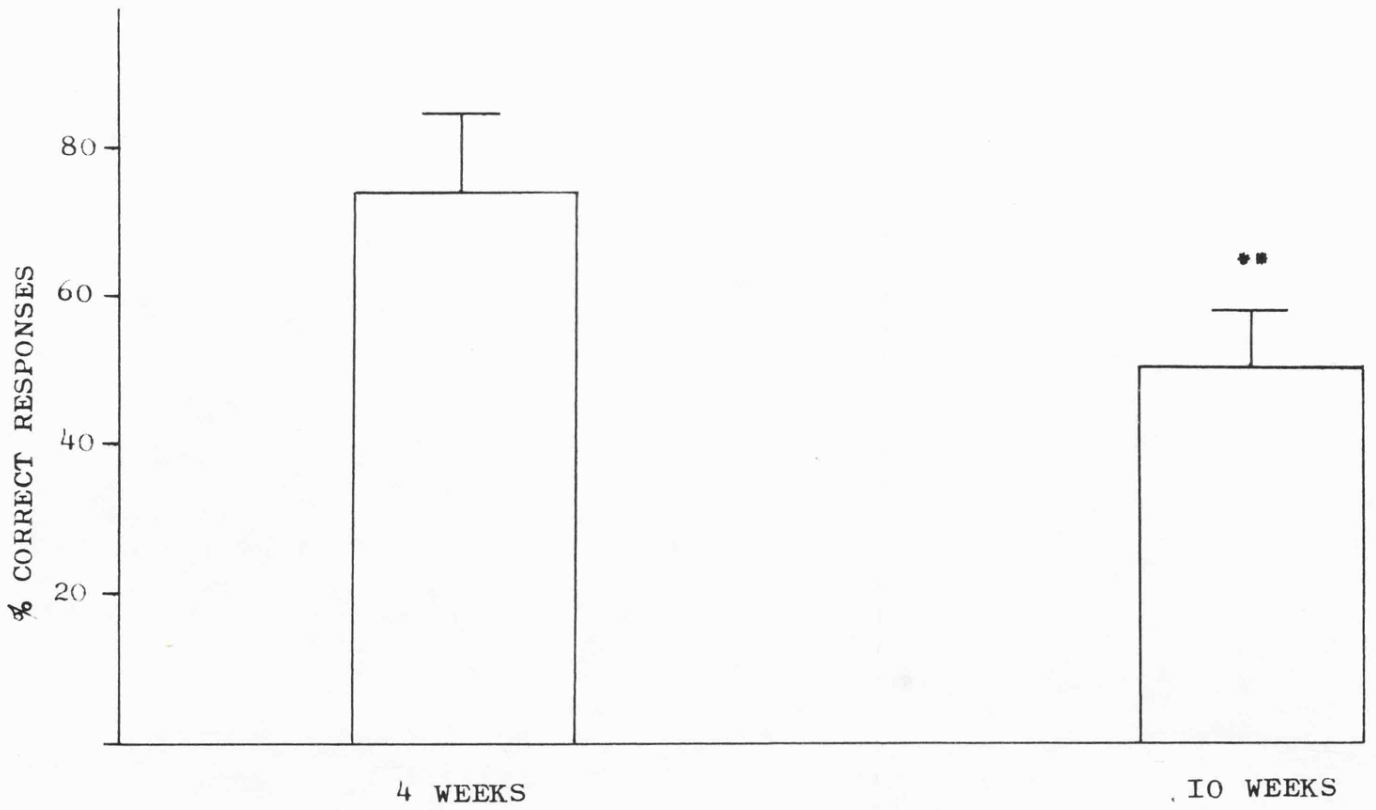


FIGURE 5

EFFECT OF FOOTSHOCK ON THE PASSIVE AVOIDANCE RESPONSE

TESTED MIDDAY. n=I2 ±S.E.*** p< ·002

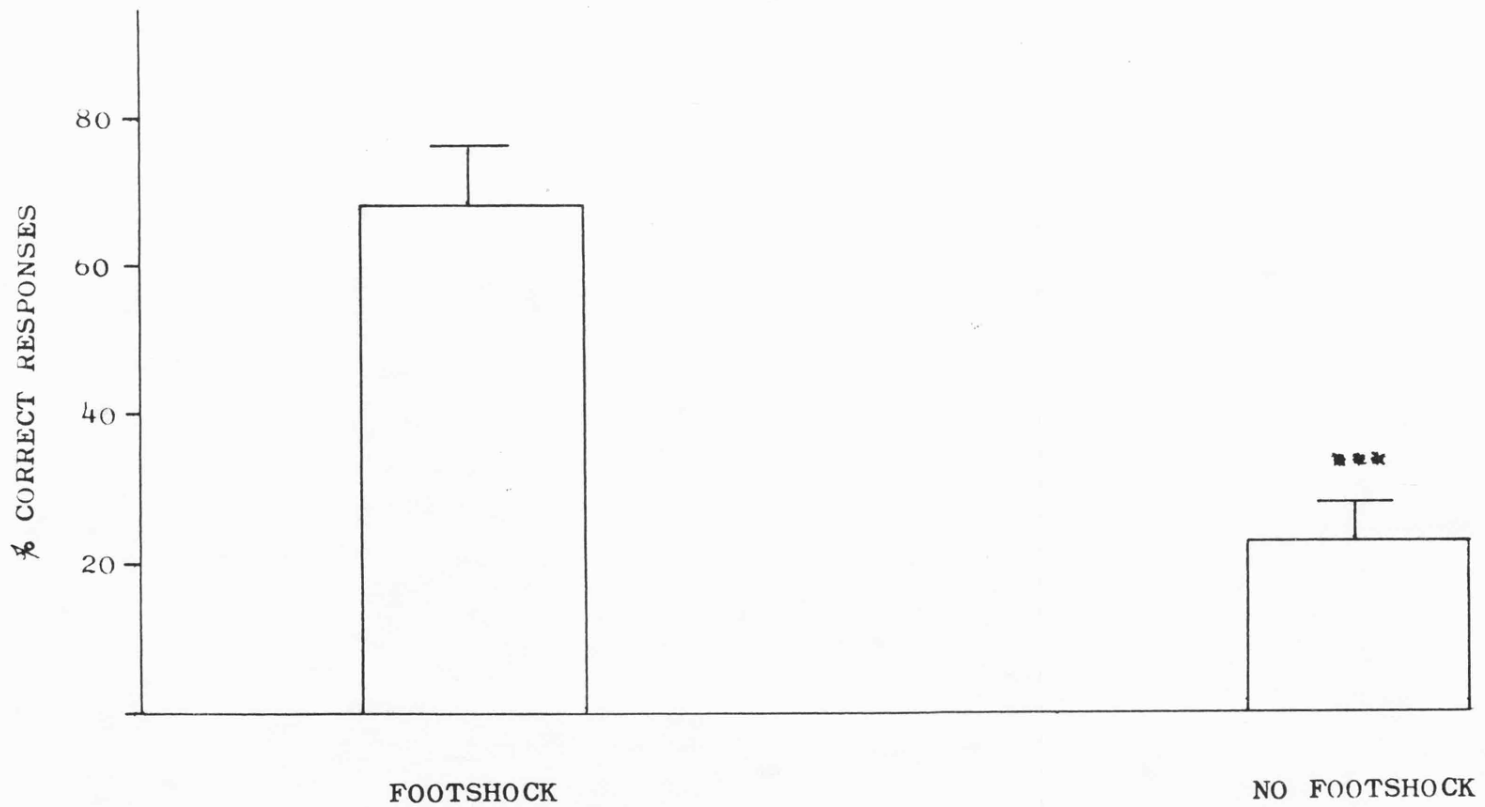


FIGURE 6

EFFECT OF INJECTION ON THE PASSIVE AVOIDANCE RESPONSE TESTED MIDDAY. n=I2 ±S.E.

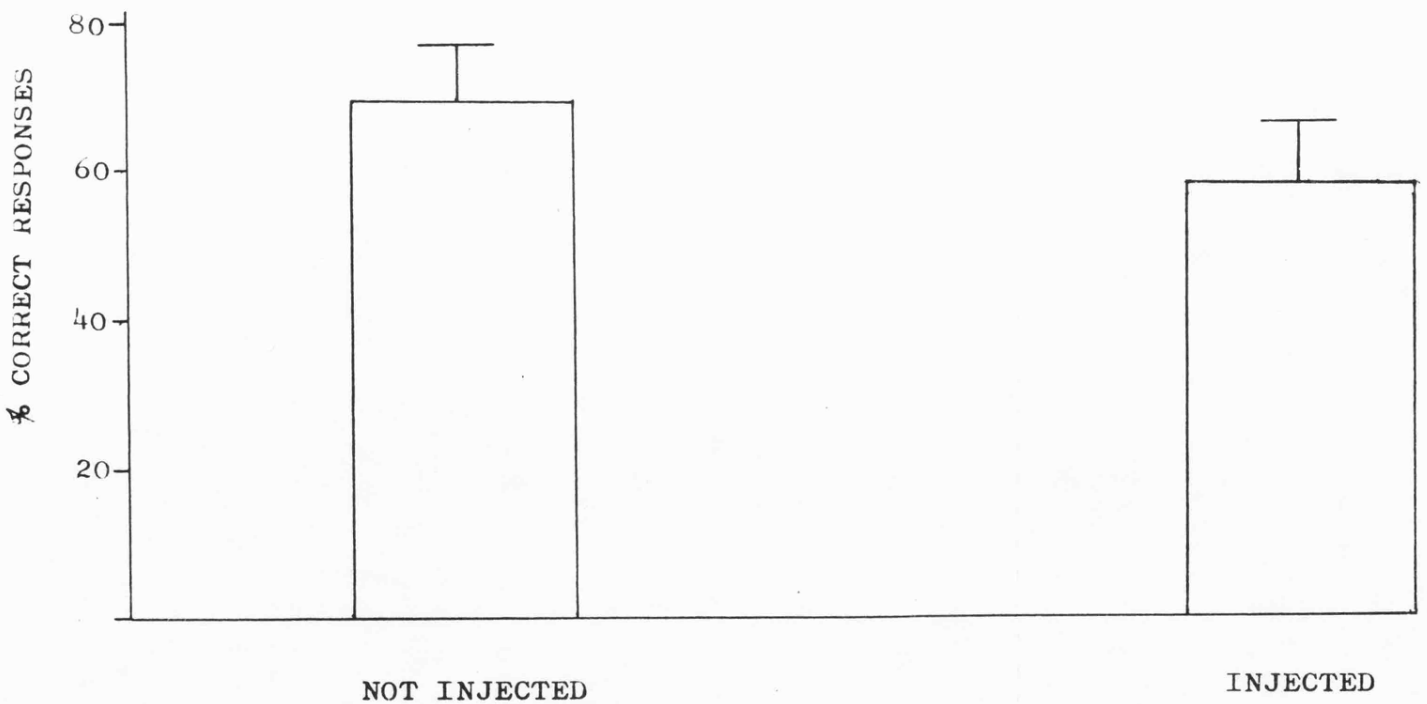


FIGURE 7.

THE EFFECT OF SOCIAL RANK ON THE PASSIVE AVOIDANCE RESPONSE. TESTED MIDDAY. n=14-16 ±S.E.

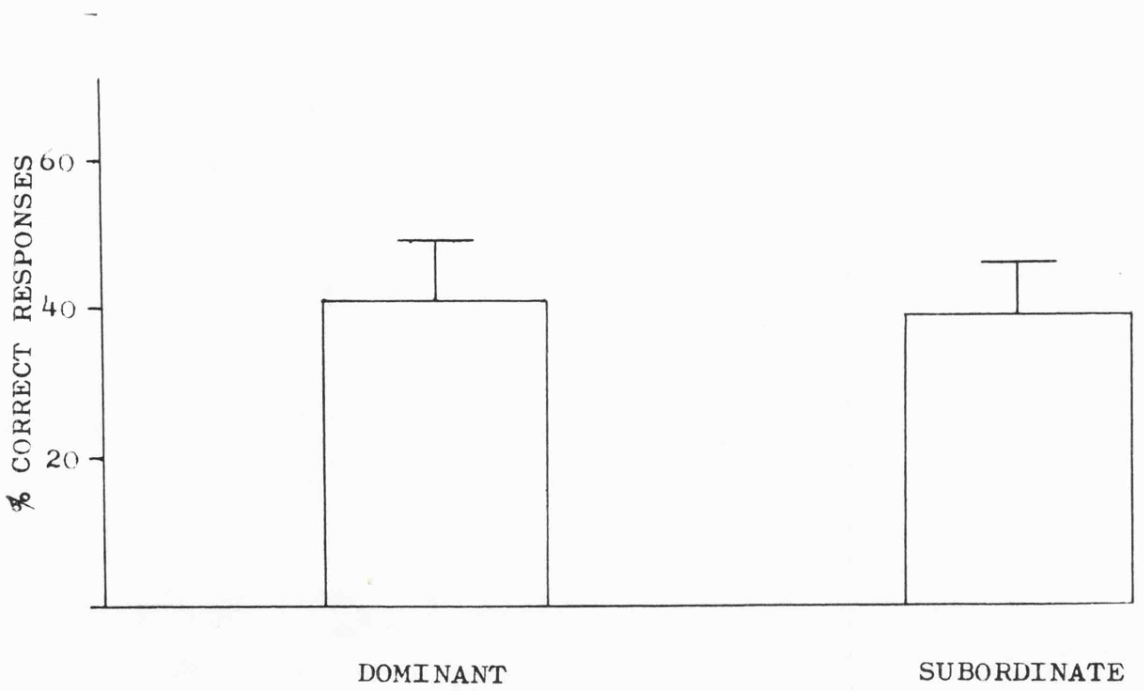


FIGURE 8

EFFECT OF BODY WEIGHT ON THE PASSIVE AVOIDANCE RESPONSE.
TESTED MIDDAY. n=12 ±S.E. * $p<.05$

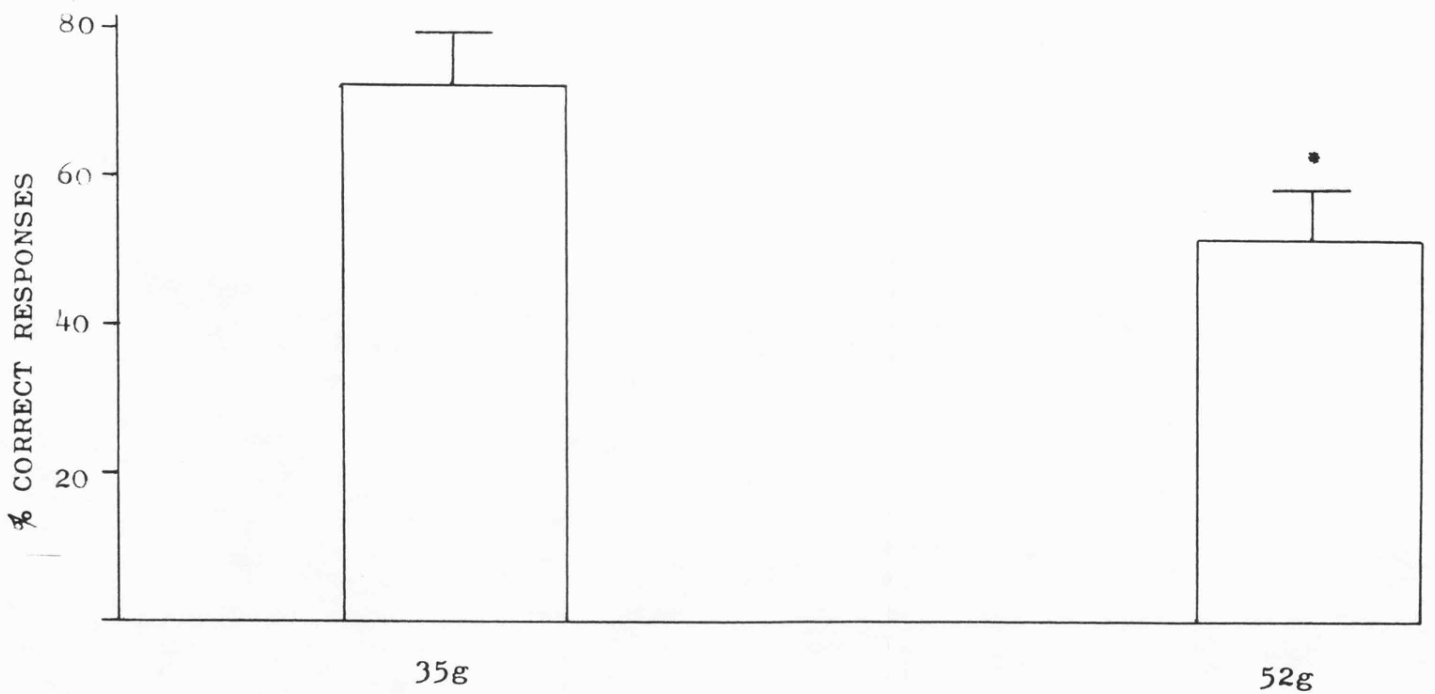


FIGURE 9

THE EFFECT OF ILLUMINATION ON THE PASSIVE AVOIDANCE RESPONSE.

n=12-15 ±S.E. ** $p<.02$

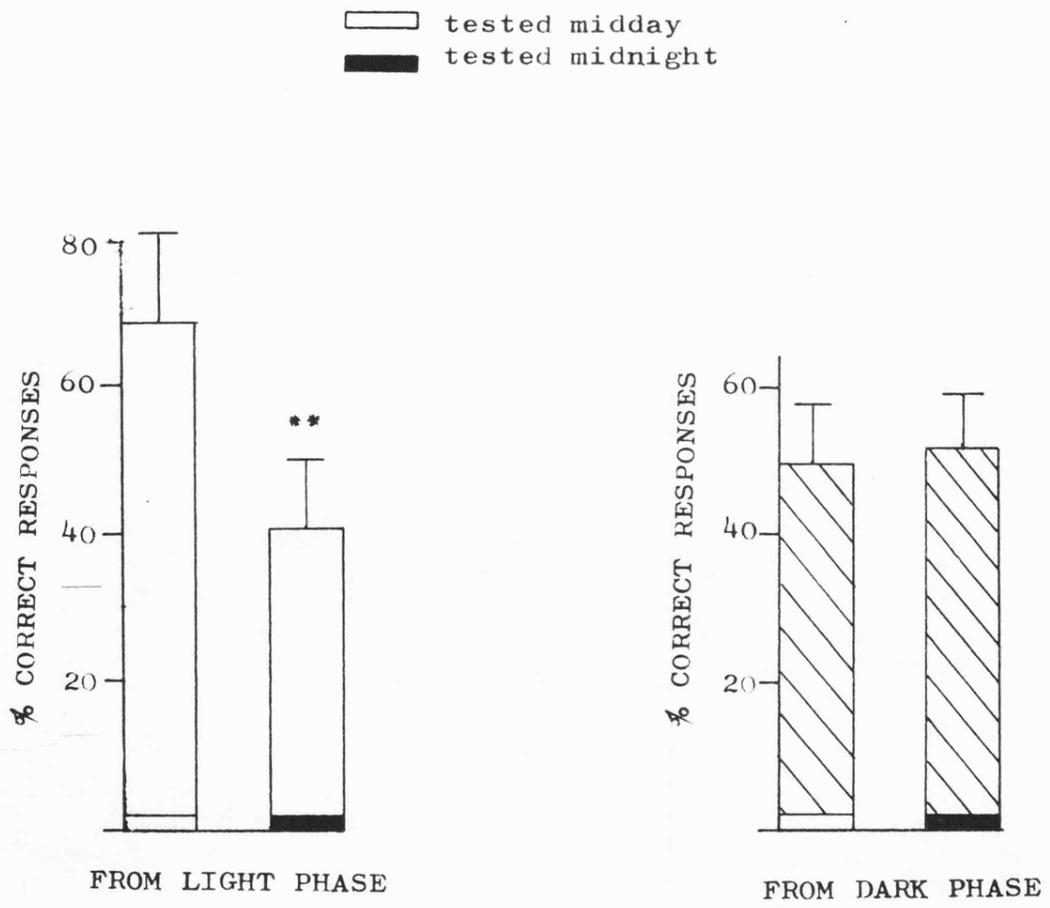
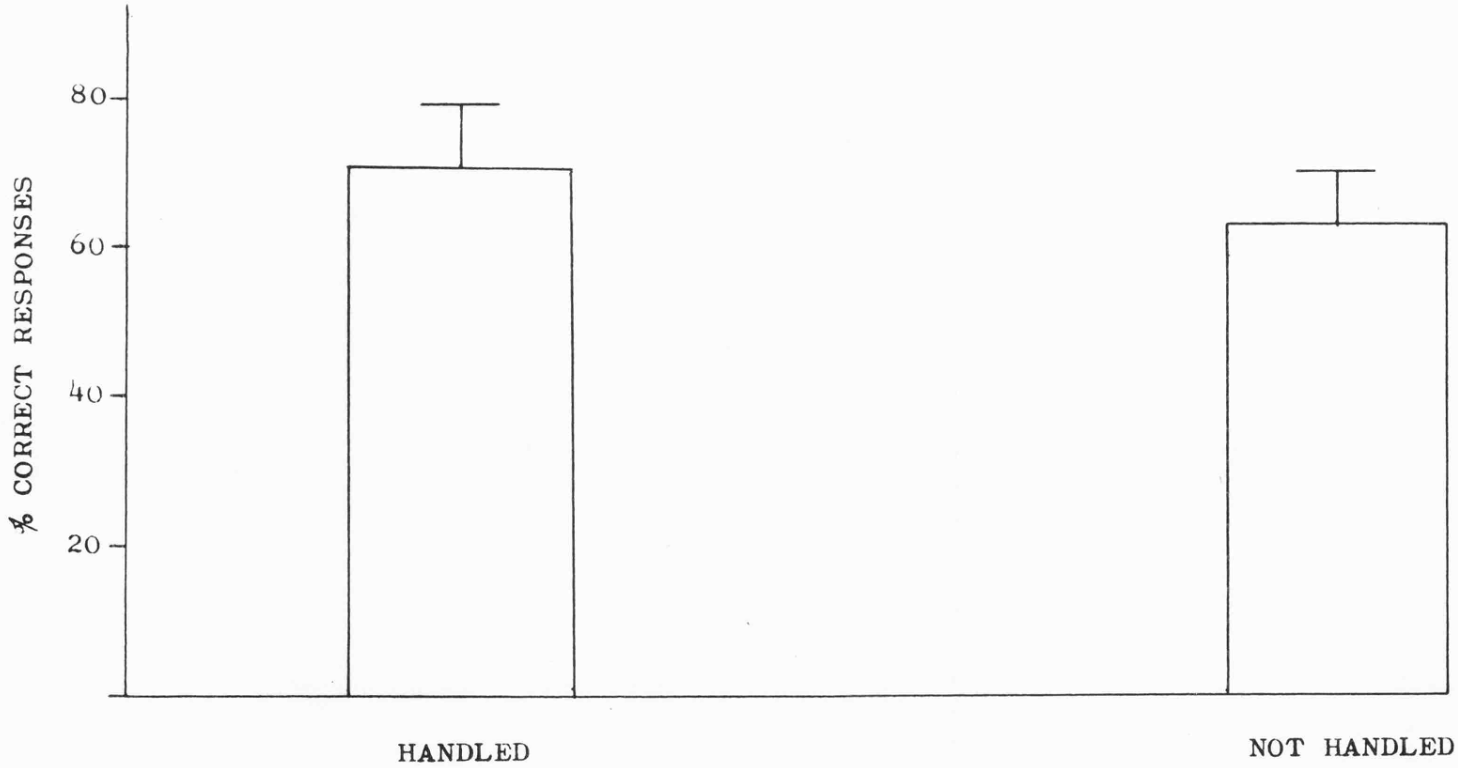


FIGURE 10

EFFECT OF DAILY HANDLING ON THE PASSIVE AVOIDANCE RESPONSE TESTED MIDDAY. n=23-25 ±S.E.



further two groups were removed from a light environment (at mid light phase), with one group (n=I2) tested under light, and the other (n=I2) in darkness under red light. Animals were 7 weeks of age.

(e) Effect of shock: One group (n=I2) was tested without shock, while a further group (n=I2) received normal electro-shock procedure. Animals were 7 weeks of age when tested.

(f) Effect of injection: One group (n=I2) received O.I ml. saline injected i.p. 30 mins. prior to each trial, while a further group (n=I2) received no injection.

(g) Effect of handling: One group (n=25) received daily handling at various times throughout the light period, for 4 weeks subsequent to weaning, while a further group (n=25) were never handled. Animals were 7 weeks of age when tested.

To avoid unnecessary experiment-duplication, inter-experimental comparisons are made in this and many subsequent cases, where comparable animals, subjected to identical experimental procedure are used.

Figure 4 shows the mean result obtained from animals of different ages, expressed as histograms. A significant difference exists between the two groups, in propensity for correct responses. The difference resulted from the greater likelihood of plate-crossing by the older animals on second exposure.

Administration of footshock elicited significantly high correct responses (fig.5), compared to those not receiving shock. Again a clear discrepancy existed between group scores on second exposure.

Administration of prior injection (fig. 6) resulted in a very slight depression of the mean score for correct responses. Though significant at the low level of $p<.10$ in the table of critical

values, this is not taken as a reliable level of significance and the information is therefore omitted.

Mean correct responses in socially dominant individuals (fig. 7) were comparable to subordinate counterparts. However the former category made more plate-crossings on both first and second trial. This is not reflected in the percentage difference score.

Difference in body weight (fig. 8) clearly elicits a significant difference in mean correct responses. This results from the greater propensity for plate-crossings on second trial in the larger animals.

Removal of subjects from the dark phase and testing them under light (fig. 9) did not measurably affect passive avoidance scores. However testing light-phase subjects in darkness did reduce the response.

Daily handling was shown not to measurably affect passive avoidance scores (fig. IO).

3.4 Discussion

3.4.I Age and body weight:

The finding of a significant depression of correct responses in older subjects confirms the age variable as an important determinant in the choice of animal subjects. As a consequence, care was taken that all future subjects were of a standardised age. Seven weeks was chosen as a convenient optimum between maturity and senescence in later experiments.

It can be seen that weight difference can also induce a similar discrepancy of result, with lighter animals giving more correct responses. It seems possible that age / weight variables may be correlated, i.e. older animals respond less appropriately by

virtue of their greater body weight (approx. 20g difference), and that response to, or sensitivity to footshock may vary directly as a function of body mass, and possibly of the skin resistance of a subject. Possible factors such as differences in activity levels, anxiety and emotionality will be later discussed. These findings appear at variance with a general appraisal of the literature, which indicates generally that younger, more naive animals respond less well in passive avoidance tasks.

3.4.2 Light

As removal of subjects from the dark phase, and testing under light did not measurably influence passive avoidance scores, the decision was made to carry out all future testing under light. This procedure has previously been adopted, as a result of similar findings in rats (Navaratnam, I973). This procedure seemed especially pertinent as removal of the animals from their light phase and testing them under darkness, apparently caused a significant depression of correct responses.

As all subsequent tests took place under light, experimental conditions could be kept constant, and inter-group comparability maintained. Further behavioural effects due to illumination differences are later discussed.

3.4.3 Social rank

The finding of no significant discrepancy between socially dominant and subordinate individuals, is perhaps surprising as dominant mice are invariably larger than subordinates. The former category did in fact show more crossings on both first and second trial, though the net result was that these two categories could not be differentiated on the basis of correct responses. The original hypothesis had been that animals formerly subjected to punitive biting (subordinates) would show their natural response to this

(freezing) when exposed to punishment stimuli. Brain (I97Ia) has postulated similarities between conditioned avoidance reactions and social aggression. He proposed that stressful attacks by a dominant could have affinities with more conventional aversive stimuli (such as electroshock), and that subordinates could act submissively when exposed to such stimuli. This hypothesis does not appear, however to be substantiated by this result.

3.4.4 Footshock

The absence of footshock, an integral component of passive avoidance experimentation, apparently causes a significant depression of mean correct responses. This result is not unexpected since the negative reinforcement of shock is the factor inducing avoidance of plate-crossing. It is most interesting, however, that non-shocked animals also displayed lowered tendency to cross on the second trial. The reverse would have been expected, due to familiarisation with the apparatus and habituation to their novel situation.

In all subsequent experiments,therefore, care was taken to ensure removal of faeces and urine between trials, with soap and water, to prevent insulation from footshock.

3.4.5 Injection

"Injection stress" apparently caused a slight depression of mean correct responses. In subsequent experiments involving drug injection, therefore, comparisons were made only with saline-injected controls only, and never with non-injected animals.

3.4.6 Handling

Though no significant difference was found between handled and non-handled mice, it must be stressed that experimenter-handling should be as infrequent and irregular as possible, to

minimise any other associated variables.

3.4.7 Practical applications of experiments

Due to the pronounced individual variability encountered in behavioural experiments, it is usually thought a good strategy to sample as widely and randomly as possible from the laboratory stock. However given this general sampling "strategy", it appears from these experiments that sampling must operate within certain confines. The following procedures were therefore initiated.

Experimental subjects were all 7 weeks of age and were randomly assigned from home cages, regardless of body weight and social rank. It was assumed that these factors would be accounted for by natural variation and the use of large sample sizes, while total randomisation would further nullify any potential experimental bias, produced by these factors. Handling was kept to a minimum, ensuring all animals received an equivalent amount, i.e. cage-cleaning once a week.

Chapter 4

VARIATION OF THE PASSIVE AVOIDANCE RESPONSE OVER 24 HOURS

4.I Introduction

Although the rhythmic nature of many psychological functions has been widely reported in the literature, 24-hour variations in mechanisms associated with learning and memory are less well-documented, though a number of studies have demonstrated the existence of variations in conditioned behaviours, as a function of the time of testing (Evans & Patton, I970; Stroebel, I967; Ghiselli & Patton, I976; Davies, et al, I973b).

The passive avoidance situation lends itself well to this kind of experiment, as it provides important information on the ability to learn and recall at different times of day, while the short duration of testing also confers compatibility with experiments involving temporal constraints, i.e. testing on the hour, every two hours.

In this and subsequent studies, the chosen duration of passive avoidance exposure was one minute, as prolongation of a test after the end of the first minute has been shown not to yield any further information and would be unnecessary (Boissier, et al, I968). I5 seconds of preliminary free exploration was allowed before each

trial.

Experiments were carried out using a total of 240 animals, 20 animals in each clock-hour sample, as use of a smaller sample-size could mask more subtle variations of the 24-hour profile. Additional reliability and accuracy were desired as these results are later used as "baseline" comparisons in later experiments.

By means of a I2-hour phase shift instituted I6 days previously, subjects from the dark phase (I800-0600 hours) could be tested during the day, and pairs of groups separated by I2 hours relative to their own light-dark cycle could be tested together at the same clock hour. This experimental design ensured that the results obtained were related to the LD cycle, and not to uncontrolled environmental factors. This system had the additional advantage of requiring the experimenter to work for I2 hours rather than 24.

Fig. II shows the mean score for plate-crossings (incorrect responses) by groups of animals at both first and second trial, over 24 hours. Both profiles peak at 0800 hours (i.e. shortly after light onset), and show mathematical conformation to a rhythm (significant, $p<.05$). Fig. I2 shows the results obtained when first and second trial scores are expressed as percentage correct responses. This method of representing the results has subsequently been adopted as it appears to constitute an index of the animals' success in recalling the previously conditioned response. The profile obtained for these results also conforms to a sinusoidal curve (significant, $p<.05$), with maximal response at mid light phase, and lowest response occurring shortly after dark onset, which interestingly coincides with the peak in other activities such as locomotor and aggressive behaviours, reviewed later in this thesis.

FIGURE II 24- HOUR VARIATION IN PLATE CROSSINGS FOR 1st. & 2nd. TRIAL
n=18-22 ±S.E. $p < .05$ (Runs test).

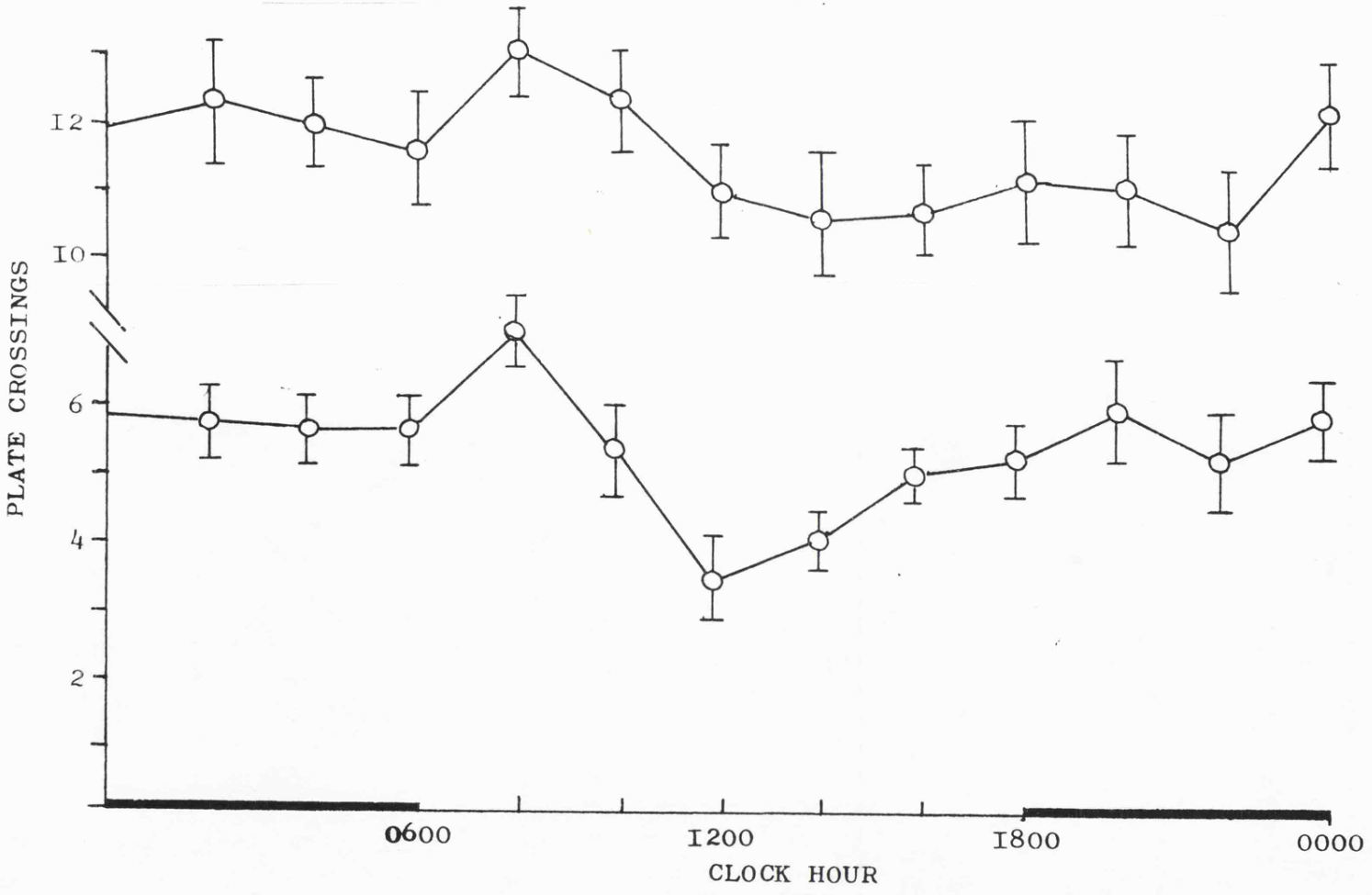
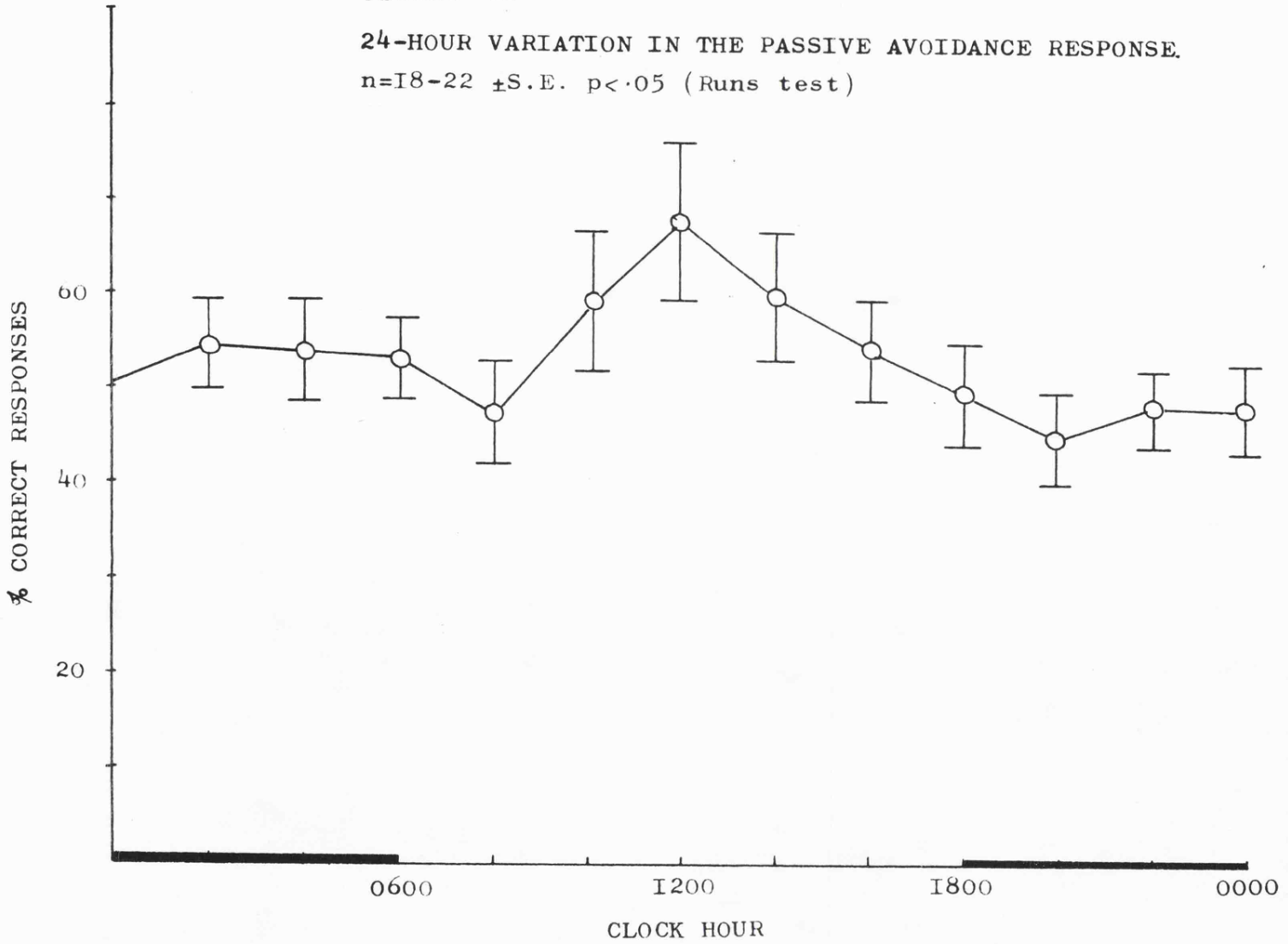


FIGURE 12

24-HOUR VARIATION IN THE PASSIVE AVOIDANCE RESPONSE.

n=18-22 ±S.E. $p<.05$ (Runs test)



4.2 Discussion

A significant variation appears to exist over 24 hours, for the passive avoidance response in laboratory mice, the first demonstration in this animal as far as is known. There remains the possibility that the variation in response stemmed from the fact that whereas some animals were taken from a dark environment and placed inside the brightly-lit apparatus, other groups were already accustomed to the light. However a preliminary study (fig.9) concluded that removal into light did not significantly affect the animals.

Another possibility to consider was the fact that individuals receiving more shock on the first trial would gain from this in terms of more learning opportunity, and therefore respond more appropriately with respect to counterparts which received less shock. However, a Spearman rank correlation test revealed rho = 0.233, on an individual's propensity to give low scores on second trials by virtue of higher scores on initial exposure. This result is not significant in the table of critical values, and it is therefore concluded that these factors do not correlate.

It seems a more likely explanation that this variation in response does reflect some aspect of learning or memory recall. There remains also the possibility that sensitivity to peripheral stimuli (such as footshock) will vary with time of day. Other psychological parameters such as arousal and anxiety levels may also show variations and are difficult to discount. Recently, Fukushima, et al (I98I) have reported the susceptibility of rats to stress to be greater during the dark phase, when animals were subjected to indirect cues of footshock. Also an anxiety state would have the effect of inhibiting learning. Thus, it is possible that any supposed

variation in cognitive processing, could reflect a variation in the animals' responsiveness to novel stimuli and the stress associated with the test situation.

This result confirms previously-reported variations in passive avoidance in rats (Davies, et al, I973b), and further confirms that successful recall of the response is greater during the animals' light phase (Sandman, et al, I97I), despite the fact that this represents the animals' inactive and dormant period.

The close resemblance of this 24-hour profile, to that of brain 5-Hydroxytryptamine levels (Ancill, et al, I970) has previously been pointed out (Davies, et al, I973b), and is later discussed.

Results from chapters 4,5 and 6 formed the basis of a paper presented to the annual meeting of the Association for Psychopharmacology, I981 (5th-7th July, Aberystwyth University), entitled...."A circadian rhythm in passive avoidance behaviour: the effect of phase shift and the benzodiazepines". These results also formed the basis of a published paper, see Childs G. & Redfern P.H., Neuropharmacology vol. 20, pp 1365-1366, 1981.

Chapter 5

THE BENZODIAZEPINES

5.I Introduction

Since the first benzodiazepine, Chlordiazepoxide, was synthesized in I955, these drugs have become the most widely used class of compounds in clinical medicine (see Blackwell, I973).

The pharmacological properties of the benzodiazepine derivatives are, though similar overall, differentiated largely on the basis of their sedative, hypnotic or anxiolytic characteristics. Most studies agree that all the benzodizepines possess, to some extent, anxiolytic, sedative-hypnotic, anti-convulsant and muscle-relaxant properties (reviewed by Greenblatt & Shader, I974). In animal studies, they have been found to possess "taming effects", and may also increase the quantity of food eaten by deprived animals (Randall & Schallack, I967).

Some investigators have claimed the benzodiazepines to possess anti-depressant properties. The difficulty involved with this assertion is a semantic one, in that no clear-cut boundary appears to exist between anxiety and depression, and in depressive disorders, symptoms are difficult to characterise as clear-cut anxiety or depression-related. Benzodiazepines are sometimes used

in the treatment of depressive illness, often in conjunction with an anti-depressant.

Perhaps the most widespread clinical application of the benzodiazepines, comes from their effectiveness in the reduction of anxiety. Their effects on brain chemistry have been the subject of much recent research, one possible mode of action being the facilitation of the activation of amino-butyric acid (GABA) receptors in the ascending reticular activating system (Costa, et al I975; Costa & Giotti, I979). As GABA is an inhibitory compound, receptor-stimulation increases inhibition and blocks both cortical and limbic arousal following stimulation of the brain-stem reticular formation. The benzodiazepines have been shown to depress electrical discharge in the septum, amygdala and hippocampus, components of the limbic system which regulate emotion in man and many animals (reviewed by Hall & Kirkpatrick, I978).

5.2 Absorption rate

The absorption-rate is a major determinant of the subjective effects of a single dose. A rapid rate of absorption is associated with rapid onset of clinical effects such as anxiety-reduction, drowsiness and relaxation. When the absorption rate is slow, these acute subjective perceptions are attenuated or eliminated (Greenblatt, et al, I977; Shader, et al, I978). The desirability of a slow or rapid absorption rate depends entirely on the clinical circumstances. When a benzodiazepine is to be taken as a hypnotic, or if the drug is used to treat anxiety, rapid absorption is desirable. On the other hand a fast absorption rate may be unfavourable if acute drug-effects are perceived as unwanted drowsiness and other sensations which may disrupt the normal lifestyle of a patient. Currently available data suggests that

diazepam and clorazepate are among the most rapidly absorbed benzodiazepines, prazepam and oxazepam the least rapidly absorbed, with lorazepam and flurazepam falling in between (reviewed by Greenblatt, et al, I98I)

5.3 Multiple-dose effects

The clinical effects of benzodiazepines during chronic or multiple-dose therapy depends in part upon the rate and extent of drug accumulation, which in turn is related to elimination half-life and clearance. The extent of accumulation is also determined by half-life as well as by the drug dosage and volume of distribution. The relative amount of accumulation, i.e. the plasma concentration at steady state, relative to that at the start of therapy becomes larger as half-life increases. Thus drugs with long half-lives accumulate extensively and slowly, whereas a short half-life implies that accumulation is not extensive and is completed rapidly.

5.4 Pharmacokinetic classification

A useful approach to the study of benzodiazepine derivatives subdivides the drugs on the basis of elimination half-life.

5.4.I Long-acting

The long-acting benzodiazepines have a net biochemical half-life of 24 hours or longer. Either the parent compound, or one of its active metabolites account for this. The half-life of chlordiazepoxide for example, generally ranges from 5-30 hours, but the half-life of the metabolites -particularly desmethyldiazepam is considerably longer (around 200 hours). Chlordiazepoxide (trade name librium), diazepam (valium), medazepam (nobrium) and clobazam (frisium), are all classified as long-acting

drugs and all are used in this study due to their low hypnotic effects at doses which alter other psychomotor parameters.

5.4.2 Intermediate to short-acting

The intermediate to short-acting benzodiazepines are characterised by half-life values ranging from 5-24 hours. Generally as half-lives become shorter, so the properties of benzodiazepines change progressively and active metabolites become uncommon. Nitrazepam, oxazepam and bromazepam are classified as derivatives with intermediate half-lives (reviewed by Greenblatt, et al, I98I).

5.4.3 Ultra-short acting

The most recently synthesized benzodiazepine derivatives have ultra-short half-life values of less than 5 hours, and are essentially non-accumulating during multiple dosage. Drugs in this category such as triazolam are used clinically as a hypnotic, while others such as midazolam are sometimes used as short- acting anaesthetic induction agents. Early published data on temazepam (normison) suggests its allocation in the short or ultra-short category (Fucella,et al, I977). However more recent studies tend to ascribe this drug nearer to the intermediate range of half-lives. However this drug has also been used in this study, to serve as a comparison with the longer-acting drugs employed.

5.5 Passive avoidance and drugs

5.5.I Introduction

The influence of drugs on learning and memory has been reviewed by numerous authors (e.g. Jarvik, I972; McGaugh & Herz, I972)

The search for pharmacological agents to enhance information-processing has been wide and varied, though complicated by the fact that different neuroanatomical regions appear to contribute to information storage in different ways. For example, physostigmine impairs retention of an avoidance task when injected into the dorsal hippocampus, whilst having no effect on the caudate putamen. Scopolamine however impairs retention in the caudate putamen and not in the hippocampus (Haycock, et al, I973). Thus in the hippocampus (a putative region involved in learning), a presumed increase in efficacy of cholinergic transmission produced an impairment, while in the caudate putamen, a presumed decrease in cholinergic transmission produced an impairment.

However there is now considerable evidence that an adrenergic mechanism is involved in memory formation and consolidation (Stein, I968; Randt, et al, I97I; Kety, I970; Dismukes & Rake, I972). For example the rate of acquisition of a conditioned avoidance task can be altered with drugs which alter amine function (Olivero, I968). Clearly a drug having the effect of altering amine levels could affect memory processes in this way. There is some evidence that diazepam (Clarke, et al, I970; Pandit, et al, I97I), flunitrazepam (Bixler, et al, I979; Dundee & George, I976), and lorazepam (Dundee, et al, I977) possess amnesic properties in humans, though it is uncertain whether the process of consolidation or retrieval is affected. It is also as yet uncertain whether these properties can be extended to all benzodiazepine derivatives.

It would appear that the benzodiazepines show both depressant and facilitatory effects in behavioural experiments. It is widely reported that they show a reduction in the acquisition of avoidance learning. Olds and Olds (I969) have proposed that the action of benzodiazepines on the hippocampus, was to depress hippocampal activity, consequently causing disinhibition which in turn cause the supression of the acquisition of the passive avoidance response. Schalleck and Thomas (I97I) found that chlordiazepoxide acted predominantly on the hippocampus by suppressing spontaneous electrical activity. Chlordiazepoxide has also been reported to reduce the frequency of, and obscure characteristic patterns of hippocampal theta activity in the rat (Iwahara & Sugimura, I970). Hence it may be suggested that the benzodiazepines supress hippocampal activity, causing disinhibition and a consequent decrease in the ability to learn.

However, numerous other workers have reported that moderate doses of benzodiazepine can actually improve active avoidance learning in rats (Bignani, et al, I97I; Sachs, et al, I966; Steiner, et al, I967), and in mice (Robichaud, et al, I973; Sansone, I975a;b;c; Sansone, et al, I972; Sansone & Renzi, I978). Favourable effects with the passive avoidance response in rats have also been obtained with chlordiazepoxide, where acquisition was improved when the response was impaired by phase shift (Davies, et al, I974).

In the following series of experiments, chlordiazepoxide was tested in mice subjected to an avoidance task requiring inhibition of movement, similar to designs proposed by Boissier (I968) and Anisman (I975). In such a situation mice can avoid an electric shock by not moving beyond a given point (outlined in chapter 2).

5.6 Administration of drugs

A major problem in later experiments was the method of drug administration. The method of dissolving the drugs in the animals' drinking water was initially chosen as it was thought imperative to cause minimal disturbance to subjects prior to passive avoidance exposure, and injecting the animals over a prolonged period would possibly introduce an additional "time cue" to which the animals could adjust. However, one obvious disadvantage of this method is the absence of an exact measure of the amount of drug administered. Spillage and degradation could account for some of the inaccuracy, though the latter was minimised by the use of light-proof drinking bottles (covered with tin foil) and daily replenishment of solutions. The volume drunk by each animal will further vary, according to body size and energy expenditure of individuals.

It is also to be expected that the rate of ingestion and consequent plasma concentration will vary with the activity / rest periods of the animals. Approximately 75% of food and water is ingested during the dark (active) phase (Zucker, I97I). Thus it is possible that the plasma concentration of a drug could also vary as a function of this daily pattern.

Before the commencement of experiments, it had to be established whether the presence of a drug in the water supply, affected the total volume of water drunk by the animals. Over a three week period, it was found that none of the drugs employed, resulted in a consumed volume which varied significantly from control (no drug present) groups. It was therefore concluded that the dissolved drugs did not measurably influence fluid intake and had not produced any "distaste" for the drinking water.

5.7 Injection

In consideration of the experimental drawbacks of the previous method of administration, it was decided to initiate further experiments involving injection. Due to the associated temporal constraints of many experiments, it was decided to inject via the intra-peritoneal route to confer a rapid rate of absorption. Unless otherwise stated, injection took place 30 mins. prior to both acquisition and recall trials.

Thus from the behavioural standpoint, the former method is perhaps more desirable, as it eliminates the likelihood of added stressors which could potentially affect later behaviour either directly or indirectly. From the pharmacological viewpoint, the latter method compares favourably, as the dose administered is a known quantity. However the use of both methods would, it was hoped, serve as a useful comparison.

5.8 Dissolving of drugs

The following benzodiazepine derivatives were employed in experiments: chlordiazepoxide (librium), medazepam (nobrium), diazepam (valium), clobazam (frisium) and temazepam (normison).

Chlordiazepoxide hydrochloride and medazepam hydrochloride were dissolved directly in distilled water, while chlordiazepoxide, medazepam and diazepam powder were dissolved in a few drops of 95% ethyl alcohol, with all solutions diluted to volume with distilled water. Clobazam powder was dissolved in a few drops of tween, while a small amount of chloroform and distilled water were used to dissolve temazepam. All solutions were diluted to the appropriate concentrations with distilled water, and all injections made i.p. in a volume of 0.Iml.

All control groups were injected with a solution

consisting of a corresponding amount of the particular solvent used, diluted to volume with saline.

5.9 Establishment of dose-response curves

Groups of animals (n=I2) were injected with various concentrations of drug, in order to confirm any facilitatory or depressant effects of each drug at different doses, compared to control groups.

Figs. I3, I4, I5, I6 and I7 show the effects of these various doses of benzodiazepine derivatives on the passive avoidance response measured at I200 hours local time. From these results, it would seem that clobazam exerted the greatest facilitatory effect (fig. I3) of all the drugs at 2.5mg/kg, while considerable locomotor impairment was noted with doses higher than this. The drug showing the least facilitation was temazepam (fig. I4). All drugs apparently induced sedation and motor impairment at the highest doses used. However clobazam produced a low response level at the lowest dose which had no effect on locomotor activity. Therefore the drug may show substantial dose-dependency in its facilitatory or depressant effects (fig. I3).

All the drugs employed induced some facilitation of the response at a particular dose level. Those considered optimal doses in enhancing the response have been employed in later experiments. These doses were chosen on the basis of whether they (a) induced strong facilitation, or (b) in the case of poor facilitation as in temazepam, the mid-range of dose which produced no motor impairment. These selected doses for subsequent experiments were: 5mg/kg (temazepam, chlordiazepoxide); 2.5mg/kg (diazepam, clobazam) and I.25mg/kg (medazepam). The subjective impression was gained that no locomotor impairment or sedative effects resulted.

FIGURE 13 DOSE-RESPONSE FOR CLOBAZAM

TESTED MIDDAY. n=12 ±S.E. ** Differs from saline controls, $p < .02$

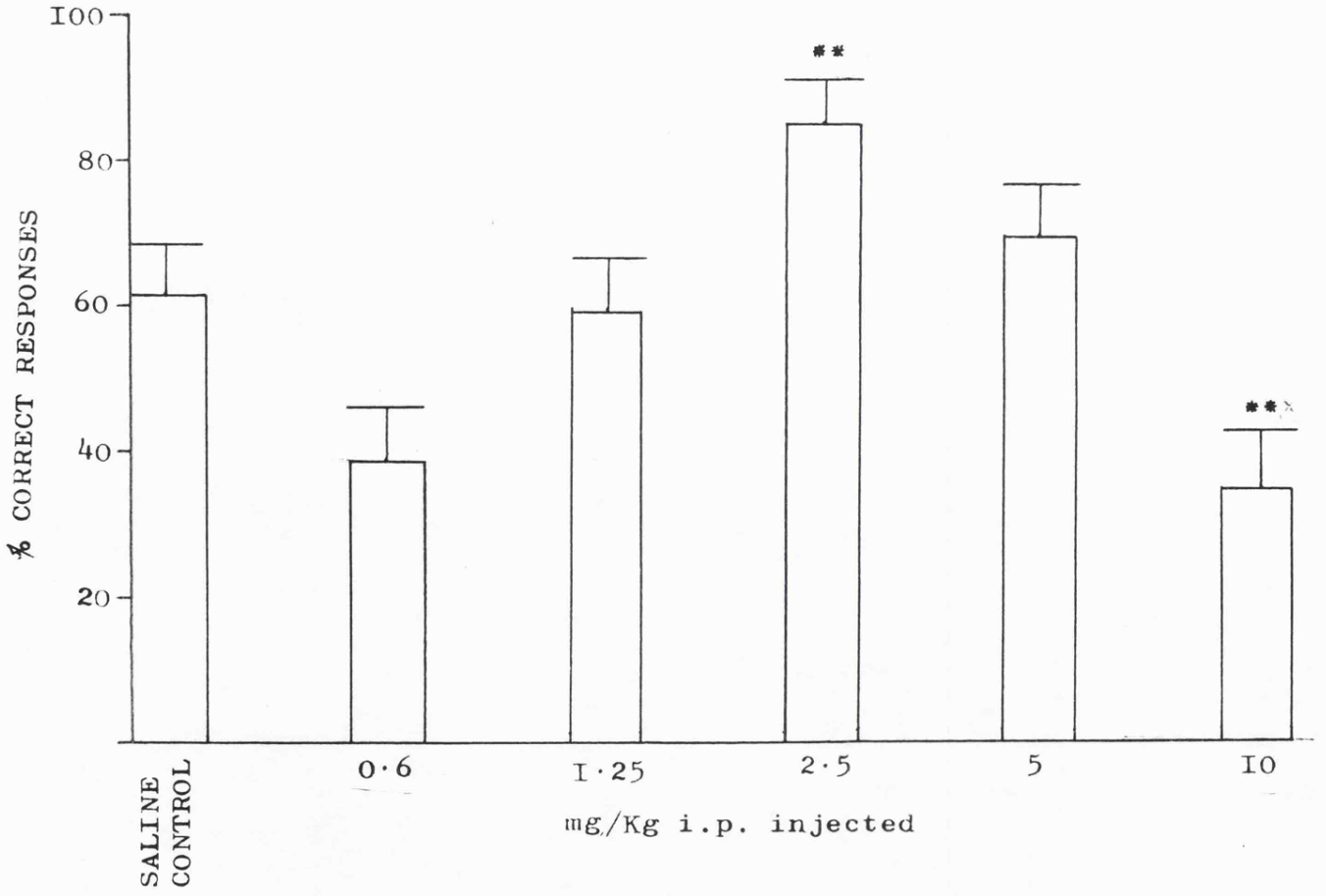


FIGURE 14 DOSE-RESPONSE FOR TEMAZEPAM TESTED MIDDAY. n=12 ±S.E.

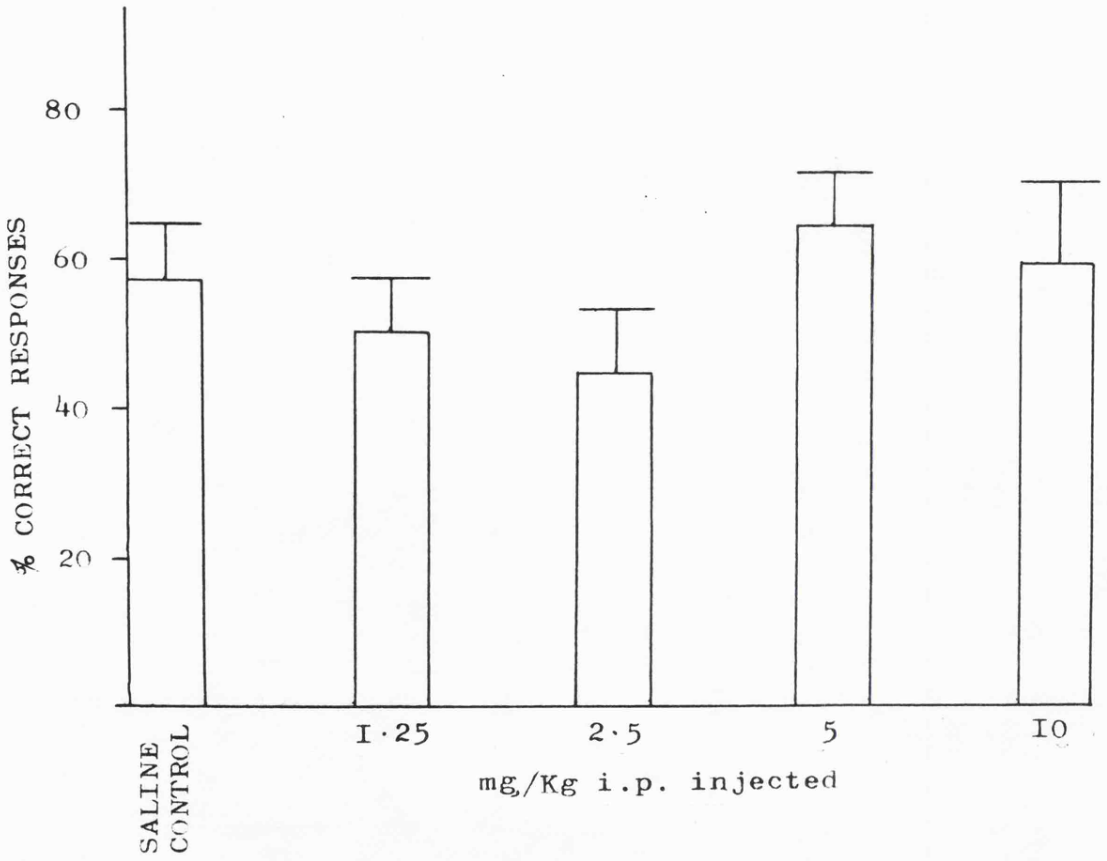


FIGURE 15 DOSE-RESPONSE FOR CHLORDIAZEPOXIDE
TESTED MIDDAY. n=12 ±S.E. ** Differs from saline controls, p< ·02

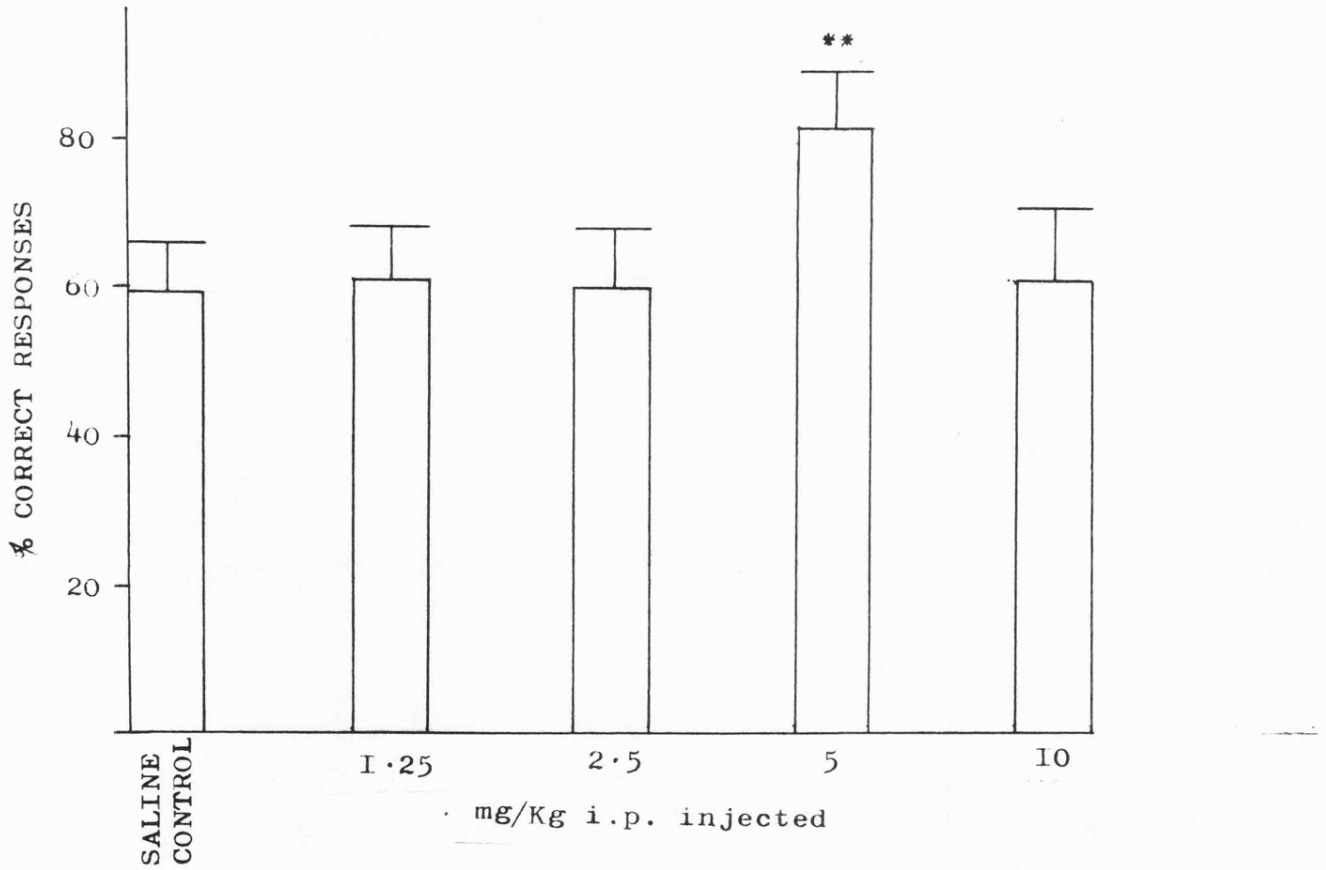


FIGURE 16 DOSE-RESPONSE FOR DIAZEPAM

TESTED MIDDAY. n=11-12 ±S.E. * Differs from saline controls, $p<.05$

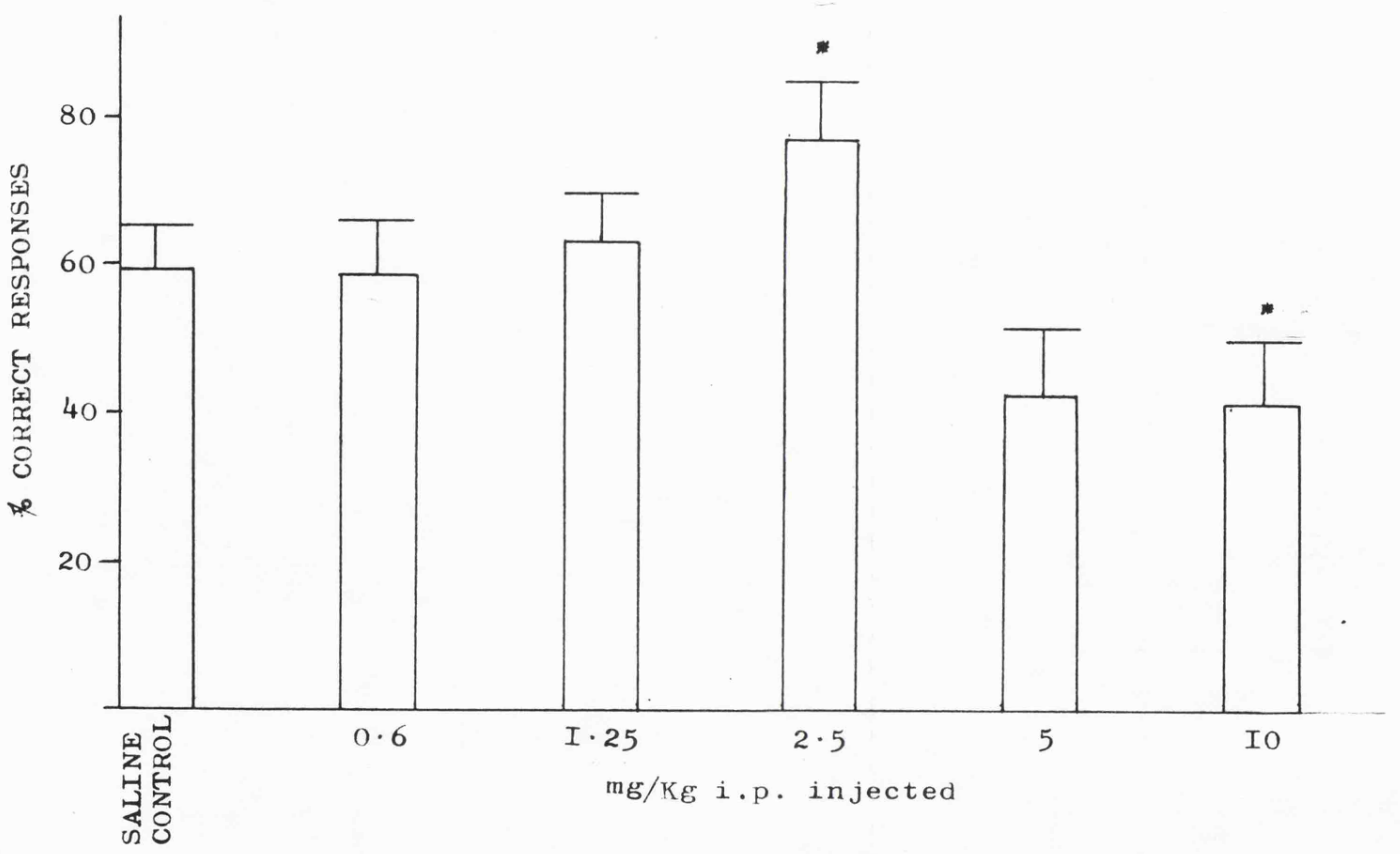
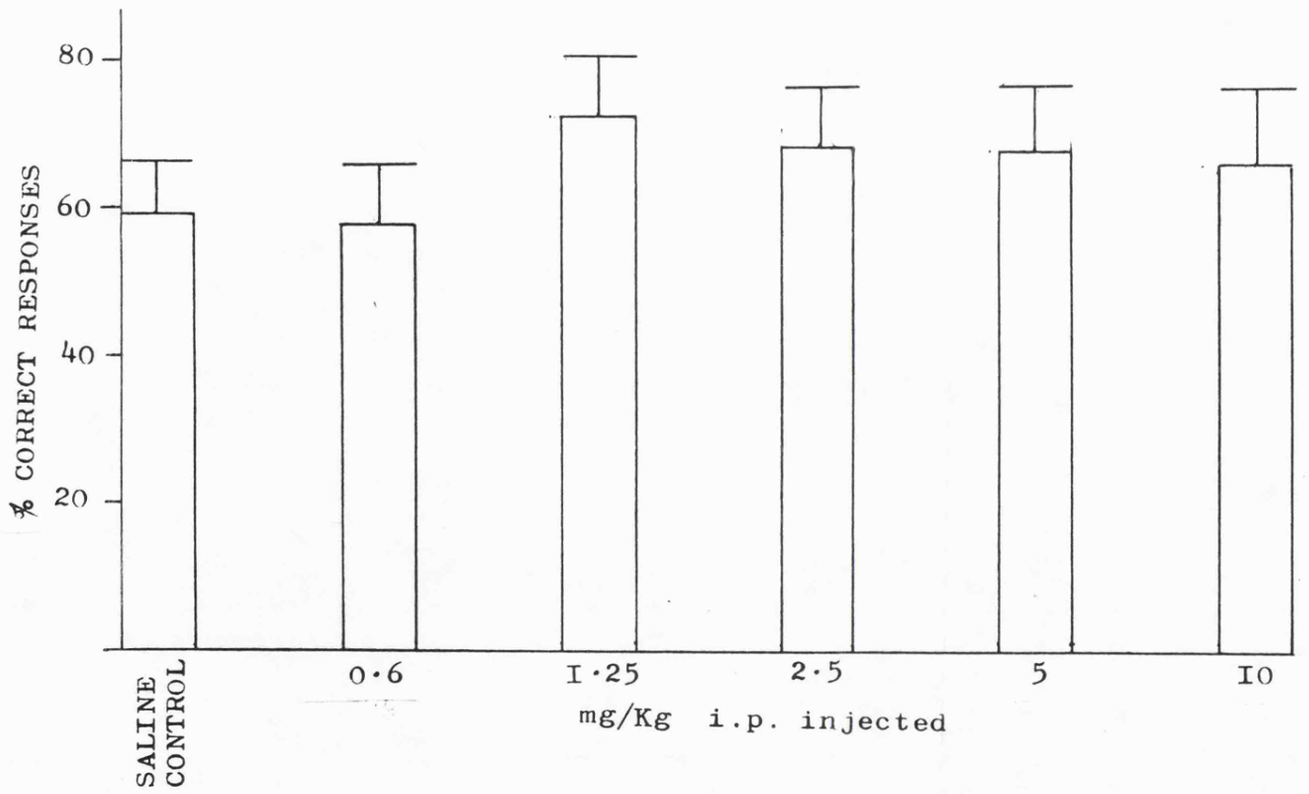


FIGURE I7 DOSE-RESPONSE FOR MEDAZEPAM TESTED MIDDAY. n=I2 ±S.E.



5.I0 Time-of-day effects of the benzodiazepines

5.I0.I Clobazam

In order to ascertain any possible variation in physiological response to drug-treatment throughout the 24-hour cycle, an initial passive avoidance experiment was conducted using clobazam, injected i.p. at the dosage found to elicit the greatest increment in response (2.5mg/kg). Fig. I8 shows the effect of the drug at four clock hours, on both acquisition and recall trials. The result confirms the reported tendency of benzodiazepines to inhibit the acquisition of the passive avoidance response, which occurred at each of the four times tested, with maximal effect at mid-light phase, compared to saline controls. However the drug evoked consistently fewer plate-crossings on retrial. This may reflect a considerable enhancement of recall success, and was not an artefact due to some groups receiving more shock (more reinforcement) on first trial, as no statistical correlation has been found for an individual's propensity to give low scores on second trial as a result of its high score on first trial.

The similarity between the profiles of the drug-treated and the non-drug treated categories may indicate that a pronounced 24-hour variation in sensitivity to clobazam does not exist. However the significant discrepancy of result in the aquisition trial at I200 hours must be noted.

Acquisition and recall scores are expressed as percentage correct responses in fig. I9. The drug-treated and non-drug-treated profiles appear superimposable, and it therefore appears that the animals' ability for successful recall is not drug-sensitive throughout 24 hours.

FIGURE I8 EFFECT OF CLOBAZAM (broken line) ON ACQUISITION (top) AND RETENTION (bottom) RESPONSE
n=I2-I3 ±S.E. ***Differs from saline controls, (unbroken line), p< · 002

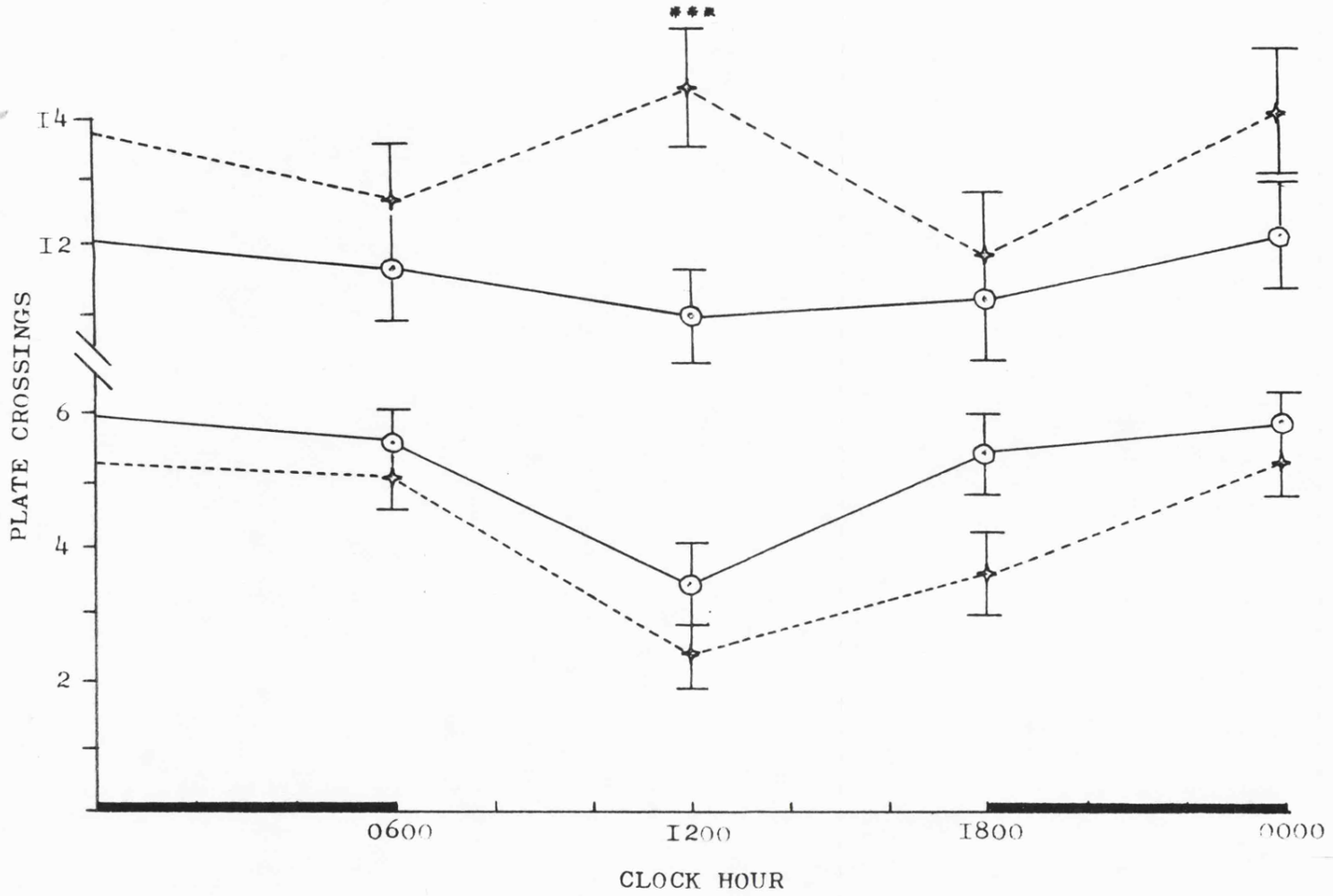


FIGURE 19 TIME-OF-DAY EFFECTS ON THE RESPONSE TO CLOBAZAM (unbroken line). n=11-12 ±S.E. * Differs from saline controls (broken line), $p<.05$

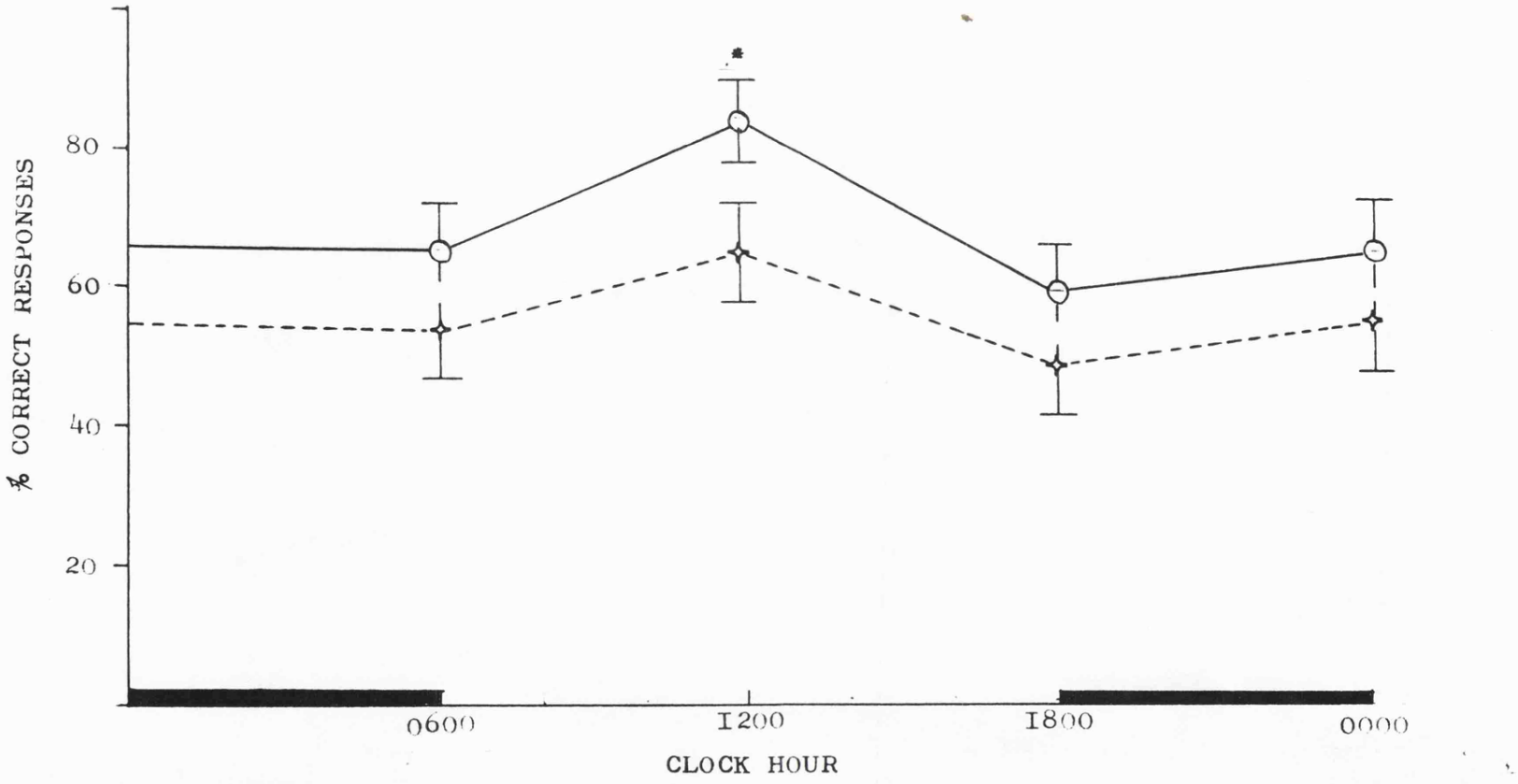


FIGURE 20 TIME-OF-DAY EFFECTS ON THE RESPONSE TO CHLORDIAZEPOXIDE.(unbroken line) n=I2 ±S.E. ** Differs from saline controls(broken line),p<·02

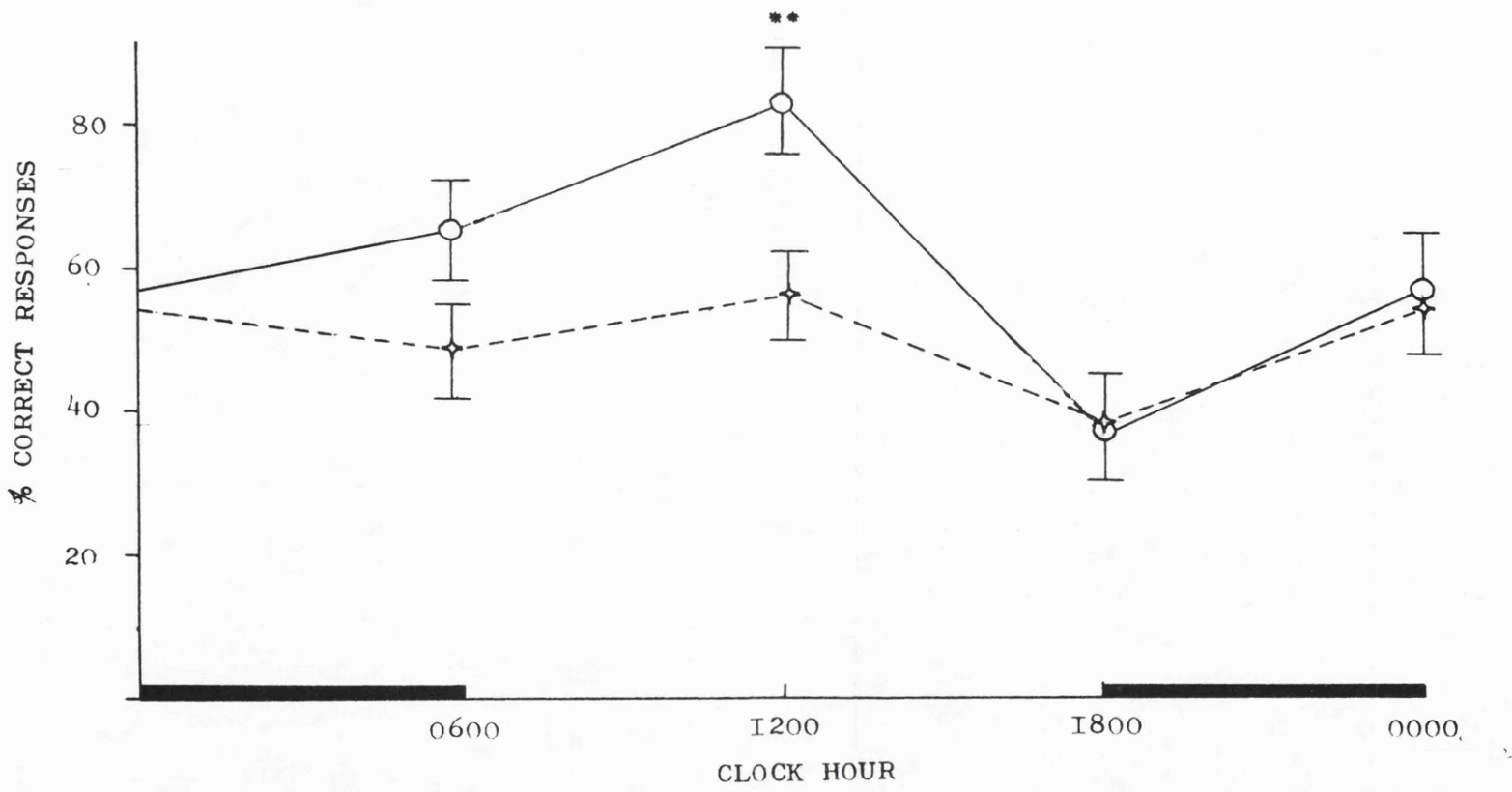


FIGURE 2I TIME-OF-DAY EFFECTS ON THE RESPONSE TO MEDAZEPAM (unbroken line).
n=I2-I4 ±S.E. * Differs from saline controls (broken line), $p < .05$

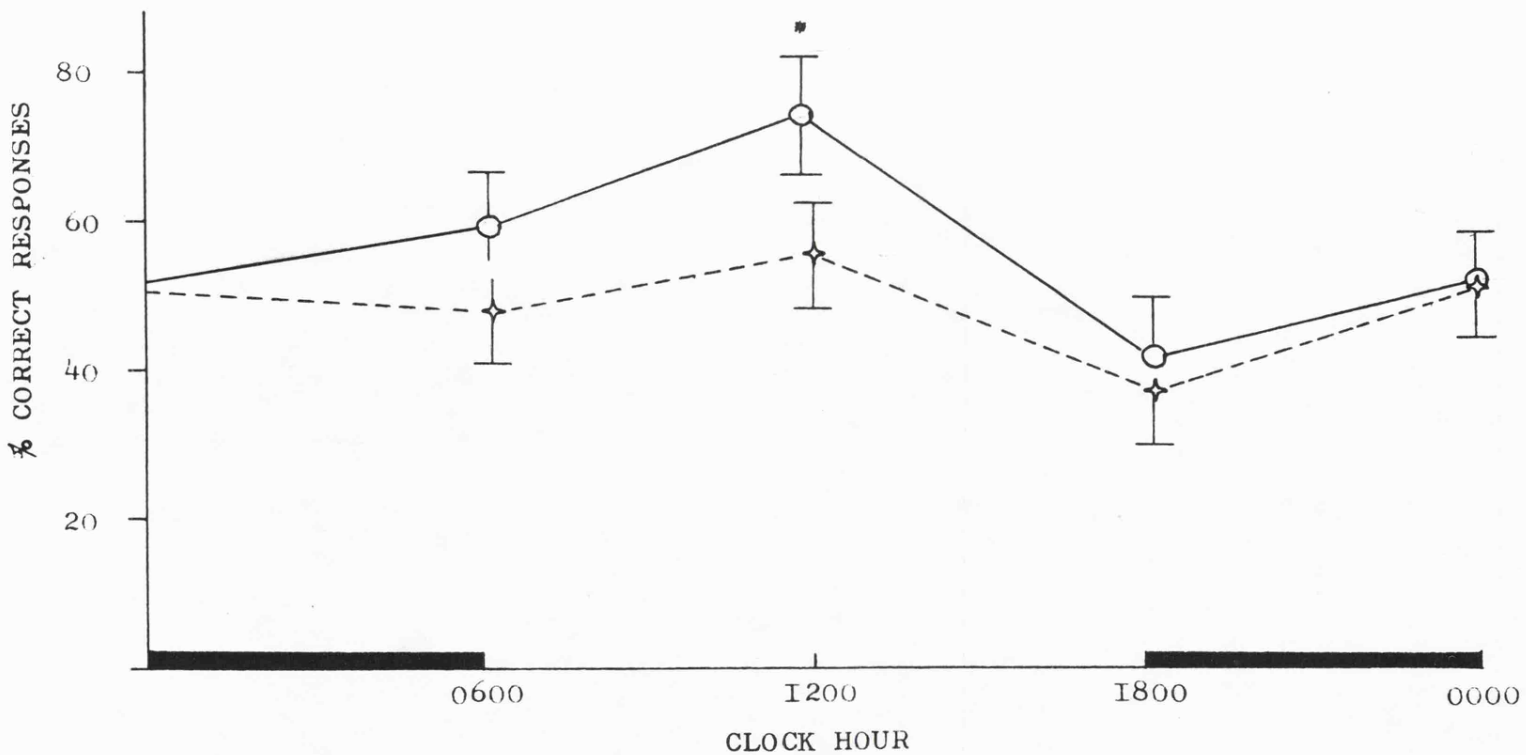


FIGURE 22 TIME-OF-DAY EFFECTS ON THE RESPONSE TO DIAZEPAM (unbroken line) n=I2 ±S.E. *** Differs from saline controls (broken line), p<·002

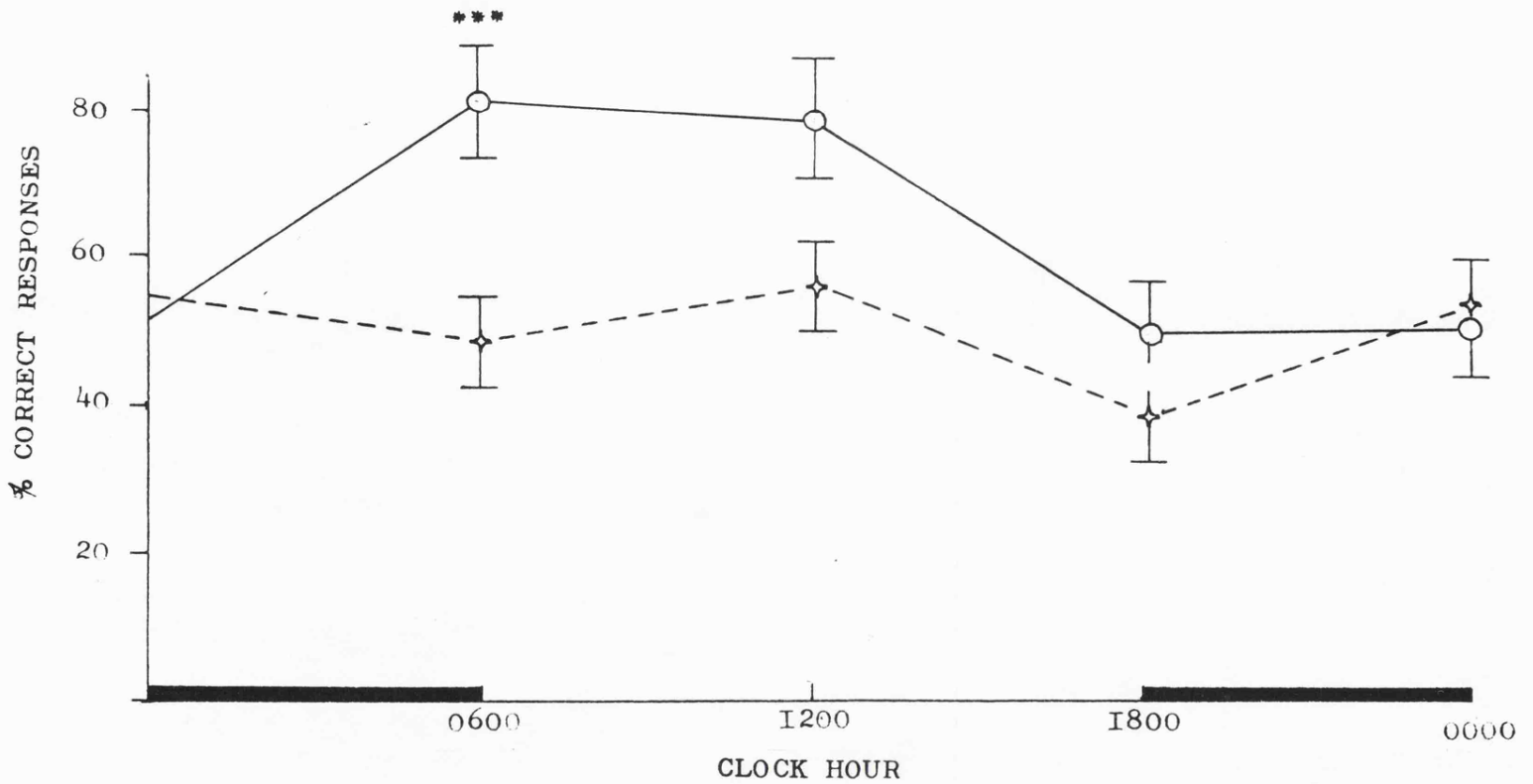


FIGURE 23 TIME-OF-DAY EFFECTS ON THE RESPONSE TO TEMAZEPAM (unbroken line). n=10-12 ±S.E. COMPARISON WITH SALINE CONTROLS (broken line)

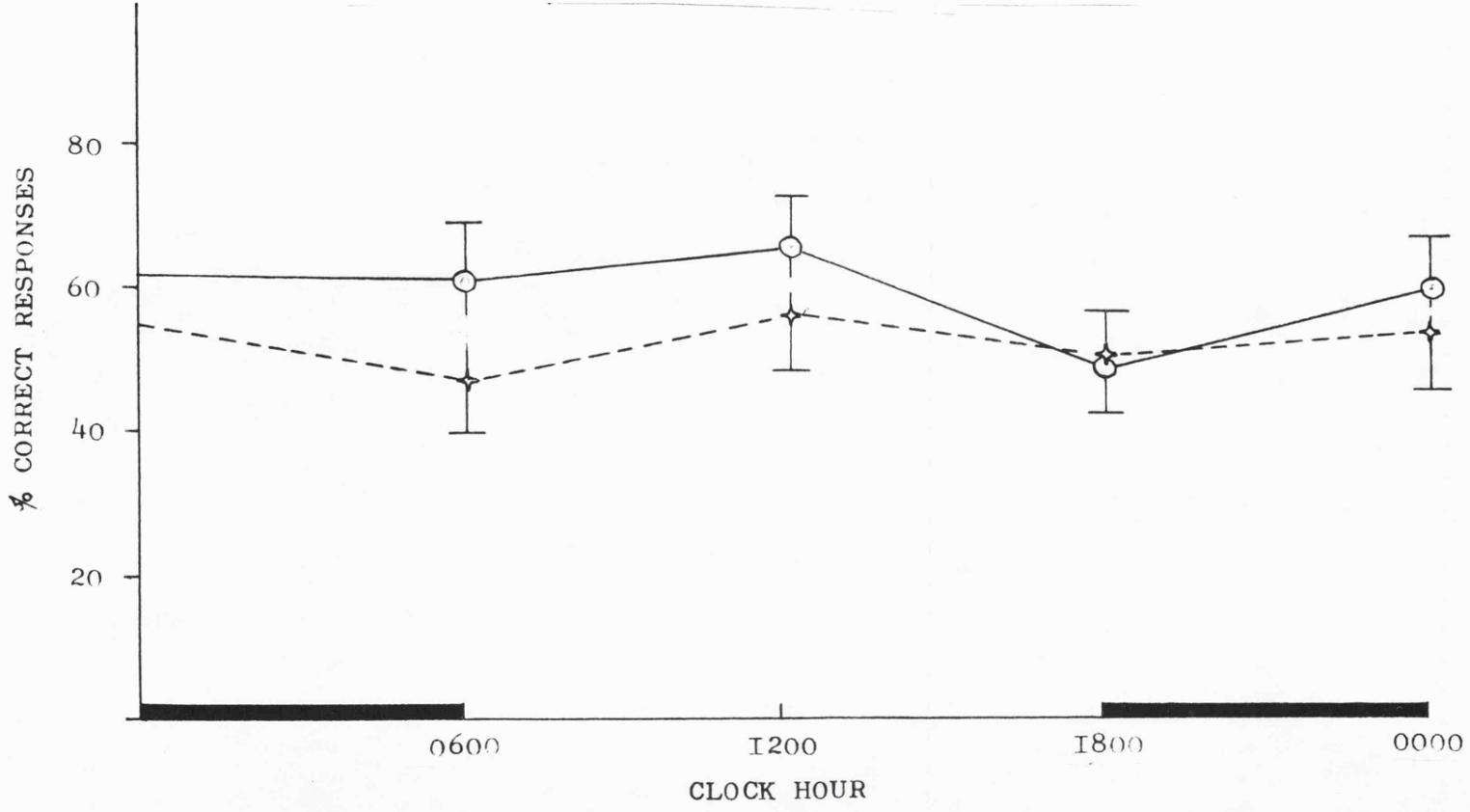
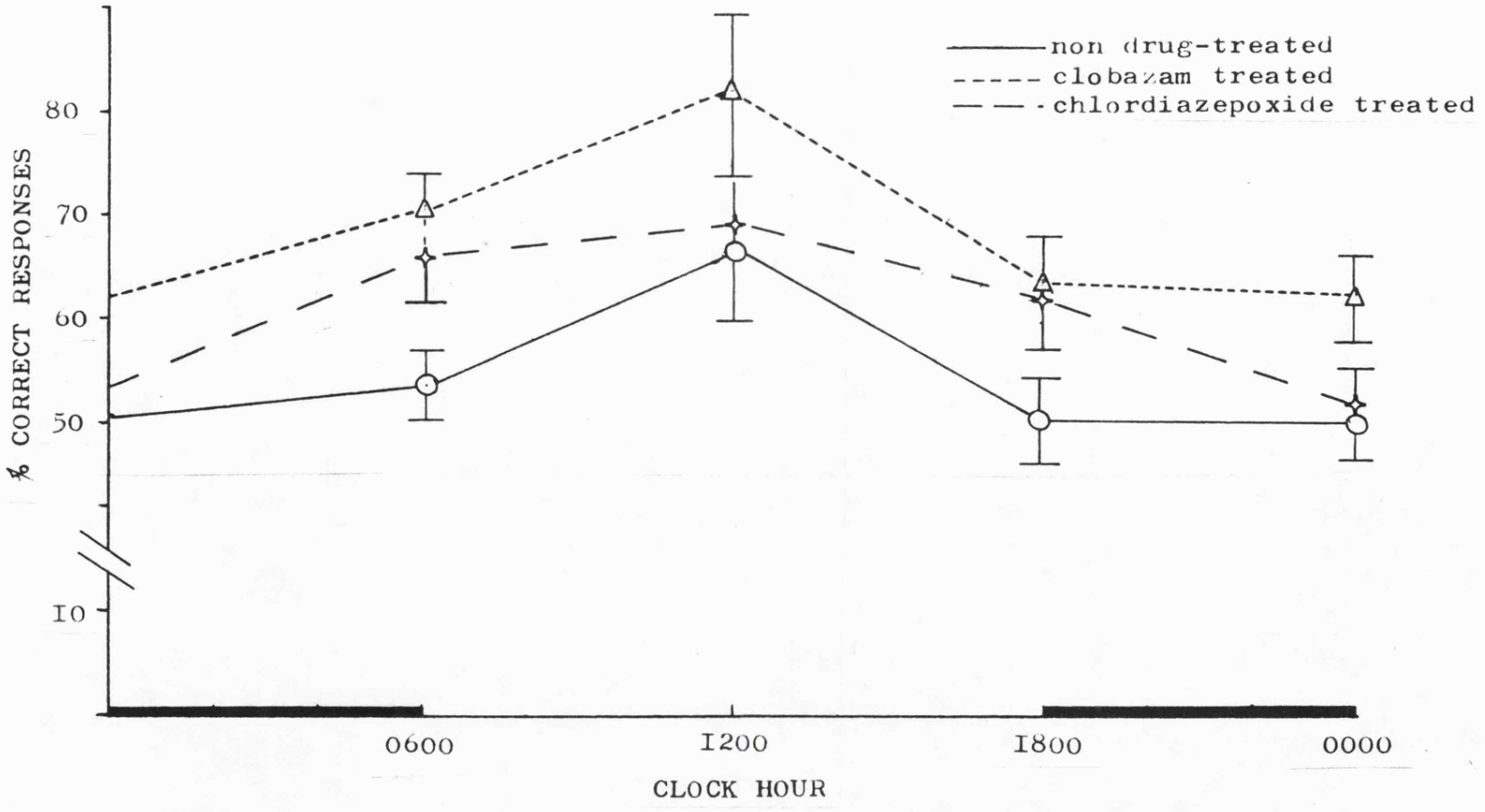


FIGURE 24 TIME-OF-DAY EFFECTS ON THE RESPONSE TO CLOBAZAM AND CHLORDIAZEPOXIDE ADMINISTERED IN THE DRINKING WATER
n=I2 ±S.E.



5.I0.2 Chlordiazepoxide, medazepam, diazepam & temazepam

Figs. 20 & 2I illustrate the profiles for chlordiazepoxide and medazepam respectively, which are very similar, and indicate that the response to drug treatment does vary throughout 24 hours. Maximum drug-effect appears to be exerted at early-mid light phase, with no effect in the initial period of darkness (I800-0000 hours).

Fig. 22 illustrates the low sensitivity to diazepam in the early dark phase. However drug-response rapidly increased at mid dark phase, rising to a maximum at light onset.

Temazepam (fig. 23) similarly shows its maximal effect in the early light phase, though this drug generally appears less successful than the other drugs in raising successful recall levels. Some locomotor impairment was noticed at the maximum dose employed. The effects noted with this drug may result from its shorter-acting nature and possibly different mode of action on the C.N.S.

Increasing the number of plate-crossings (incorrect responses) in the first trial, was a notable feature of all these drugs, as was the facilitatory effect noted in the second exposure.

The results confirm the overall tendency of the benzodiazepines to modify the passive avoidance response. They also show the drugs to be time-dependent in their effects, with a tendency to show their greatest influence in the early light phase, with some derivatives causing greater facilitation than others.

5.II Administration of drugs via the drinking water

A comparative study was undertaken, again with monitoring at 4 clock hours,to dtermine the effect of drugs when dissolved in the animals' drinking water. This method, however

involved the difficulty of determining the appropriate concentration to be used, which would approximately correspond to that used in injection. The mean daily water consumption was first ascertained as 8.8mls. / 24 hours for each mouse of 35g. body weight, with the inherent inaccuracy due to spillage, evaporation and degradation.

It can be seen that the optimal doses of 5mg/kg (chlordiazepoxide), 2.5mg/kg (clobazam) and I.25mg/kg (medazepam) translate to approximately 475ug, 237ug and II8ug respectively per unit volume, as an hourly dose administered in the drinking water. However preliminary experiments revealed these conversions to be inadvisable as sedation and ataxia was induced. A preliminary study employing doses of I00ug/ml chlordiazepoxide, 25ug medazepam and I5 ug/ml clobazam did however yield results which corresponded to those which would have been expected had the animals been subjected to injection with optimal doses, which did not impair locomotor activity or any other apparent changes in the appearance of the animals' movements.

It therefore seems that in order to acheive the optimum plasma level of drug, which enhances passive avoidance behaviour without impairing other functions, animals must drink for approximately 5 hours (chlordiazepoxide & medazepam) and approximately I.7 hours (clobazam). This may reflect the characteristics of the drug in question, such as different absorption rates.

Previous investigators (Navaratnam, I973) have demonstrated I00ug/ml. to enhance retention of the passive avoidance response in rats, when administered in the drinking water for at least 24 hours prior to exposure to the apparatus. Though involving drawbacks such as possible influence by active metabolites of the

parent drug, and the time-of-day variation in water consumption, it was decided to adopt this method. Fig. 24 illustrates the response to clobazam and chlordiazepoxide at four clock hours following exposure to the drugs in the drinking water for 24 hours prior to first and second trials. The results show clobazam to exert a significantly greater facilitatory effect at mid light phase, compared to chlordiazepoxide, with some enhancement of the response at all times tested. The results for clobazam concur closely with those for injected animals (fig. I9). However some variance of result must be noted in the case of chlordiazepoxide, between "water treated" (fig. 24) and injected animals (fig. 20). This may indicate that drug-plasma levels do not correspond, or, more importantly that the method of drug administration is a major determinant of result, and that behavioural effects may result from injection "stress".

Chapter 6

PHASE SHIFT AND PASSIVE AVOIDANCE

6.I Introduction

It has been very well established during the last few years, either by experimental phase shift of an artificial zietgeber against local time (Aschoff, I965), or by multiple time-zone travel (Gerritzen, et al, I969), that rapid displacement of temporal cues causes transient desynchronization in biological processes. This desynchronization may have such manifestations as adverse effects on psychological and behavioural function. A review of this evidence presented in chapter I indicates that the time taken to resynchronize varies between individuals and with the particular rhythm under study.

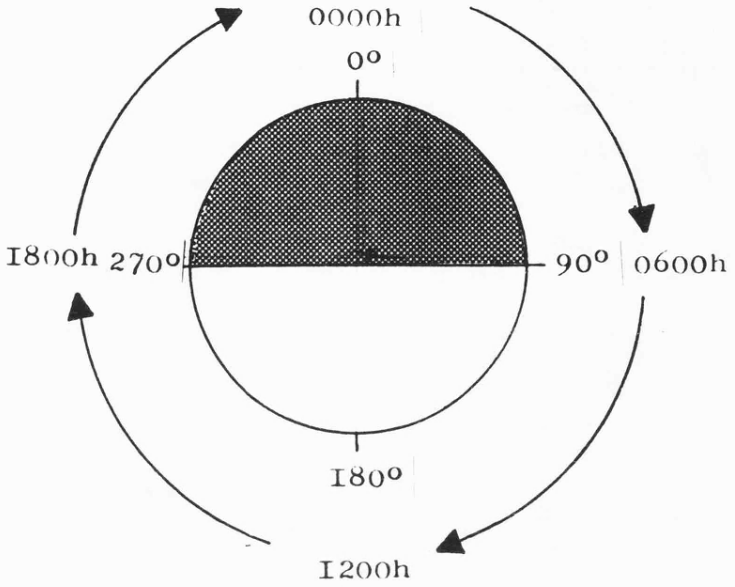
As previously reported in chapter I, a phase advance is generally thought to induce a greater magnitude of desynchronization than a delay, and an advance of 6 hours has been found to provide as great a disruption to the passive avoidance response in rats as a I2-hour phase shift, though requiring a shorter resynchronization period (Navaratnam, I973).

Because of its proven disruptive effect on rats, a 6-hour advance was instituted in the following experiments and thereafter unless otherwise stated. Also the relatively short resynchronization period lent itself more readily to experiments where availability of animals was always a problem, as a lower number of post-phase-shift daily samplings were required.

In all experiments involving a phase-shift of external zietgebers, a shift in the animals' LD cycle was made at I200 hours local time (mid light phase, biological time), and brought forward to the I800 hours dark onset, corresponding to a 90^{o} West-East transfer (see fig. 25) without the act of physical translocation and "flight stress". Thus I200 hours local time became I800 hours relative to the animals. Daily sampling then took place (unless

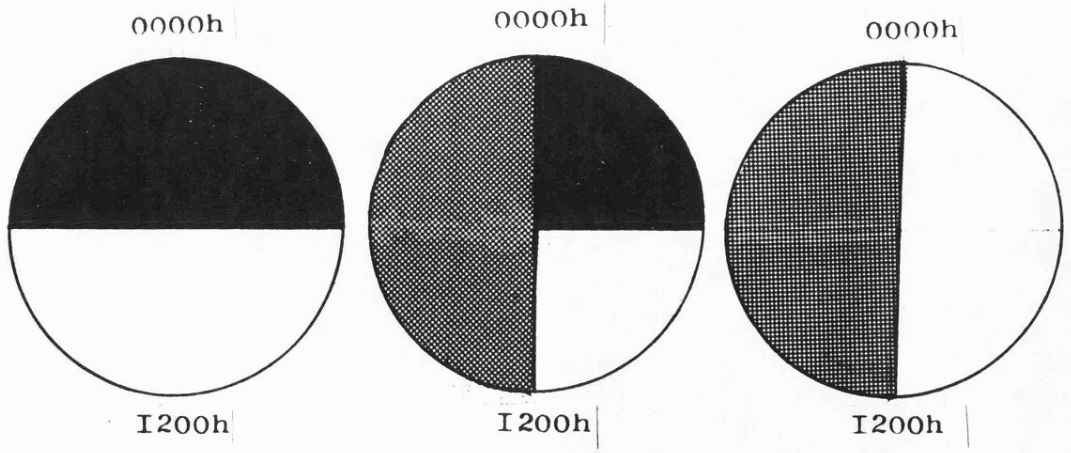
FIGURE 25

DIAGRAMMATICAL REPRESENTATION OF PHASE SHIFT (6 hrs advance)



(a)

This indicates the correspondence of a 360° phase angle to 24 hours. It can be seen that an advance of 6 hours constitutes 90° of travel.



(b) represents the pre-phase-shift LD I2:I2 cycle

(c) following phase shift at I200h, it can be seen that animals receive 6 hrs illumination in the total 24hr period

(d) represents the post phase-shift LD I2:I2 cycle.

FIGURE 26 THE EFFECT OF A 6 HOUR (90°) PHASE ADVANCE ON PAR. % CORRECT RESPONSES BEFORE PHASE SHIFT AND FOR 8 DAYS THEREAFTER IS COMPARED TO THE 1800 RESPONSE BEFORE PHASE SHIFT. TESTED 1200
n=12-20 ±S.E. ** Differs p<.02

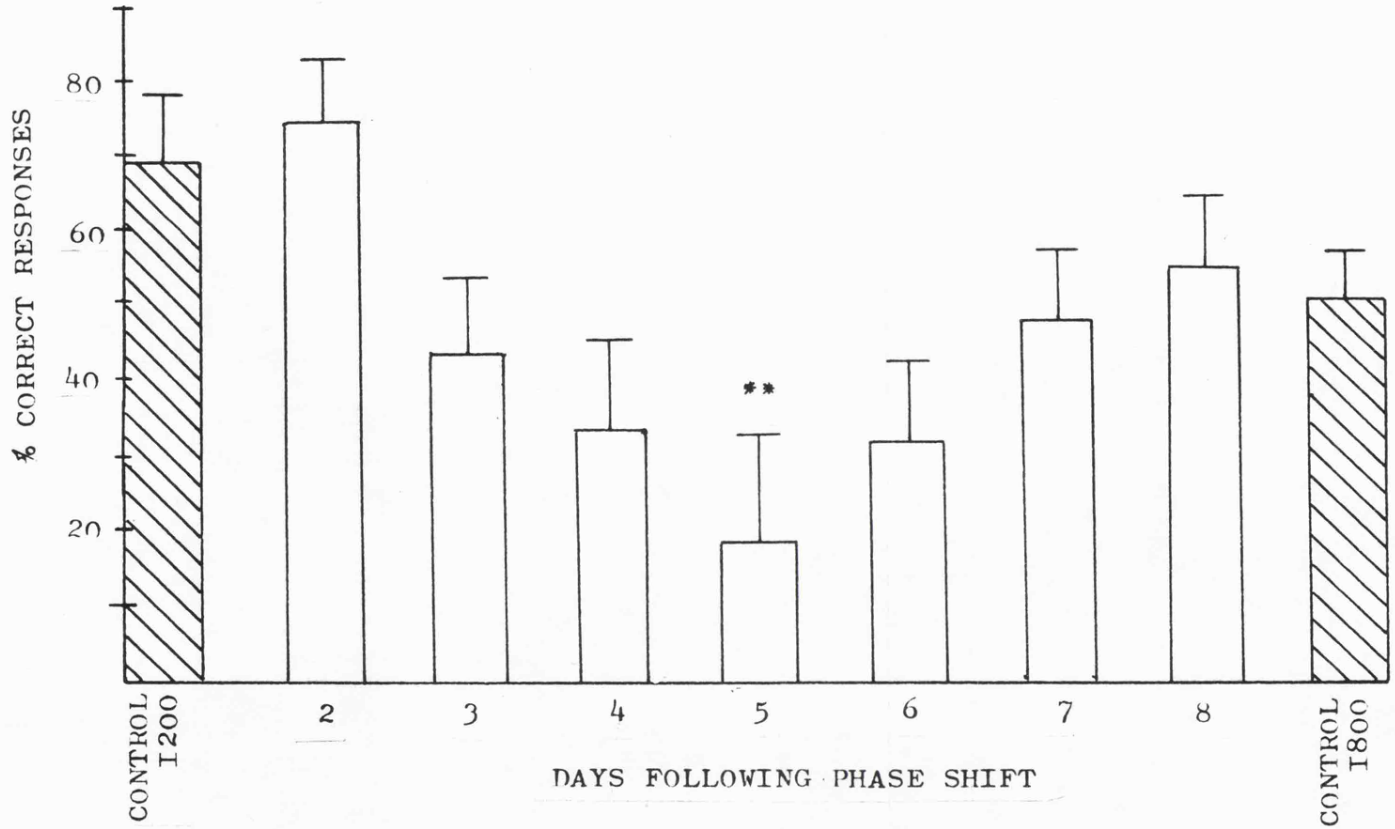


FIGURE 27 THE EFFECT OF A 6 HOUR (90°) PHASE ADVANCE ON PAR. % CORRECT RESPONSES BEFORE PHASE SHIFT AND FOR 8 DAYS THEREAFTER IS COMPARED TO THE 0600 RESPONSE BEFORE PHASE SHIFT. TESTED 0000
n=12-20 ±S.E. ** Differs p<·02, * p<·05

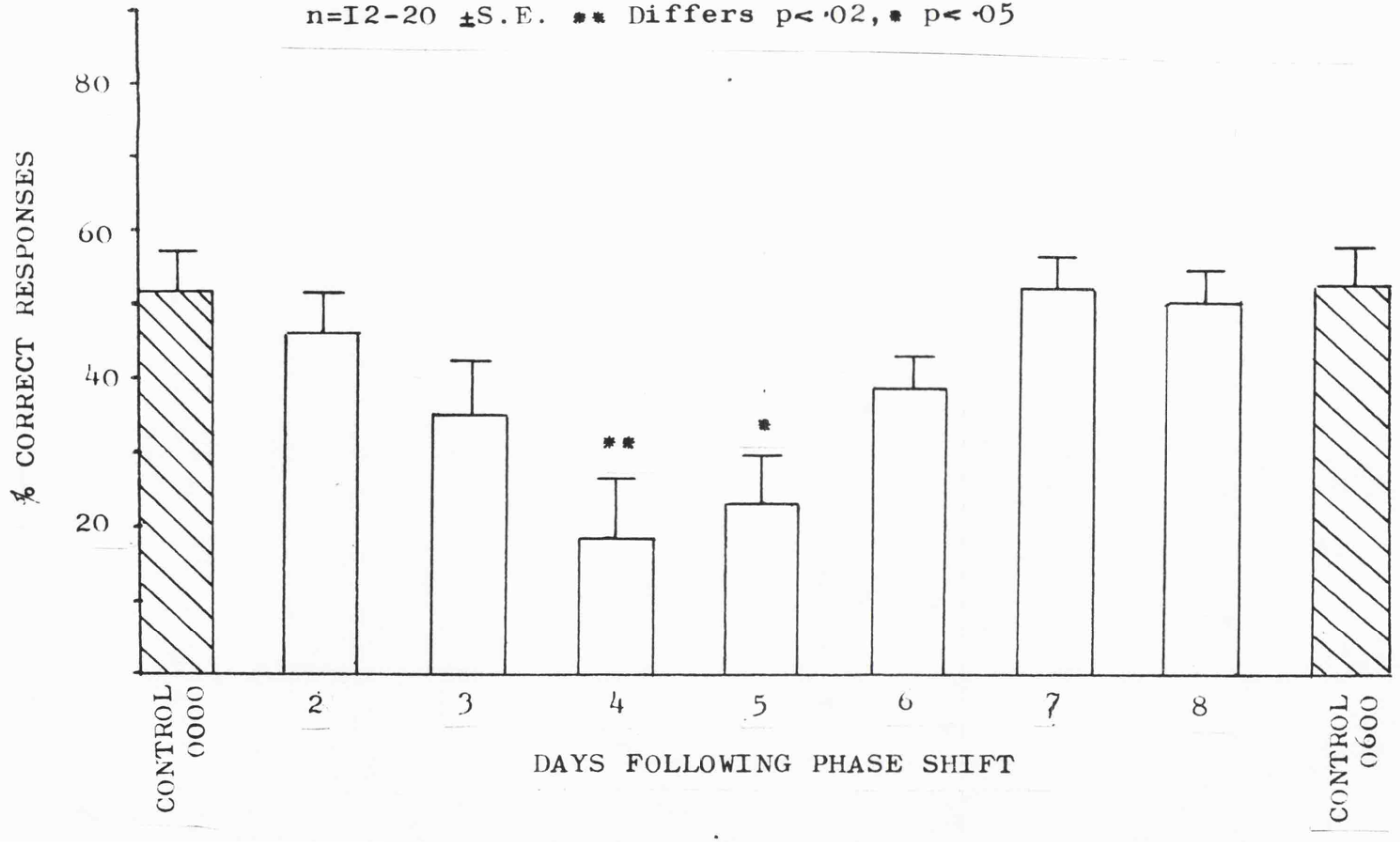


FIGURE 28 THE EFFECT OF 100ug CHLORDIAZEPOXIDE ON PHASE SHIFT (shaded columns) COMPARED TO PHASE SHIFTED CONTROLS (blank columns) TESTED 1200. n=12-20 ±S.E. *** Differs p<·002, ** p<·02, * p<·05

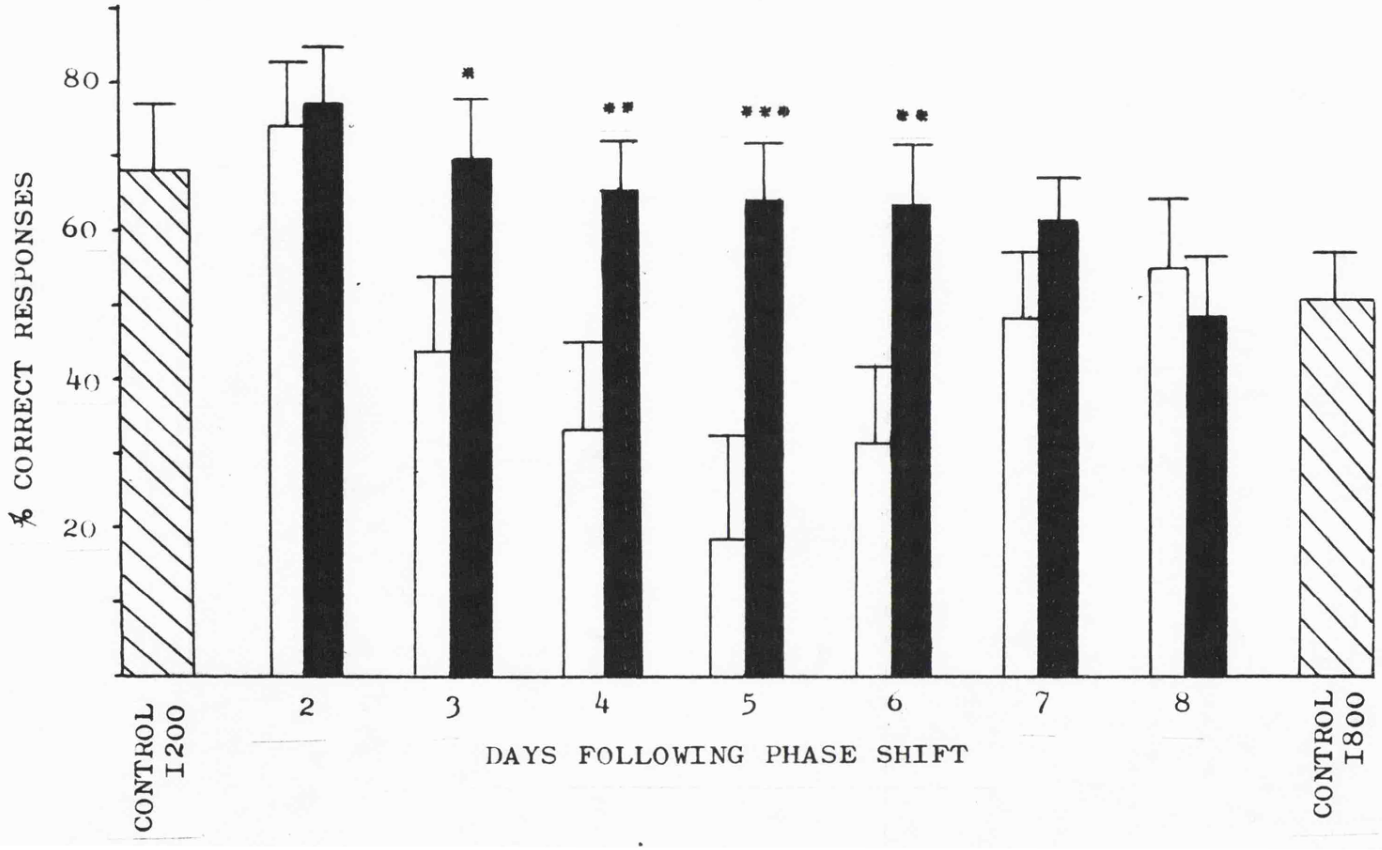


FIGURE 29 THE EFFECT OF 25ug MEDAZEPAM ON PHASE SHIFT (shaded columns) COMPARED TO PHASE SHIFTED CONTROLS (blank columns) TESTED I200. n=I2-20 ±S.E. ** Differs p< ·02

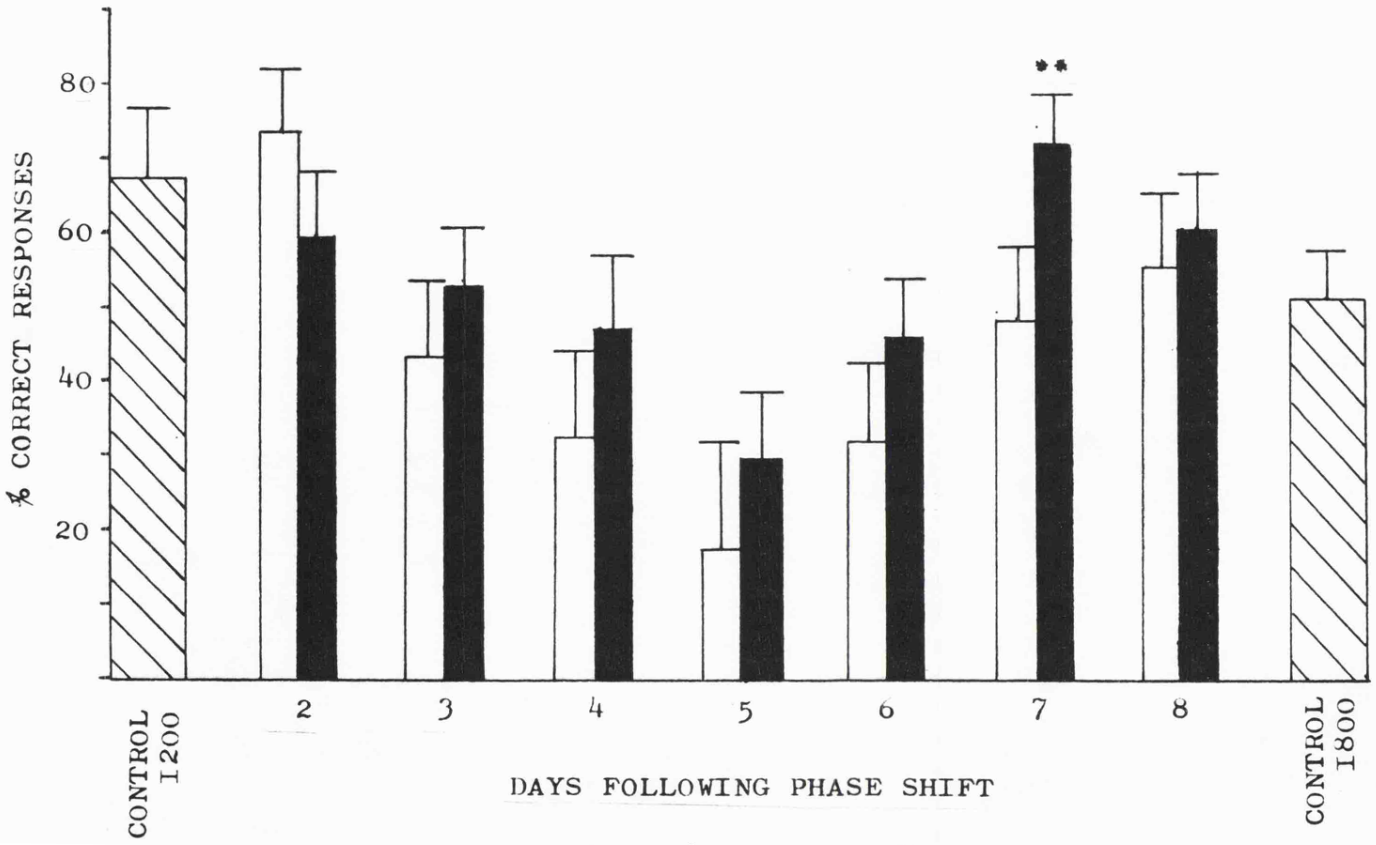


FIGURE 30 THE EFFECT OF I5ug CLOBAZAM ON PHASE SHIFT (shaded columns) COMPARED TO PHASE SHIFTED CONTROLS (blank columns) TESTED I200. n=II-20 ±S.E. *** Differs p< ·002, ** p< ·02.

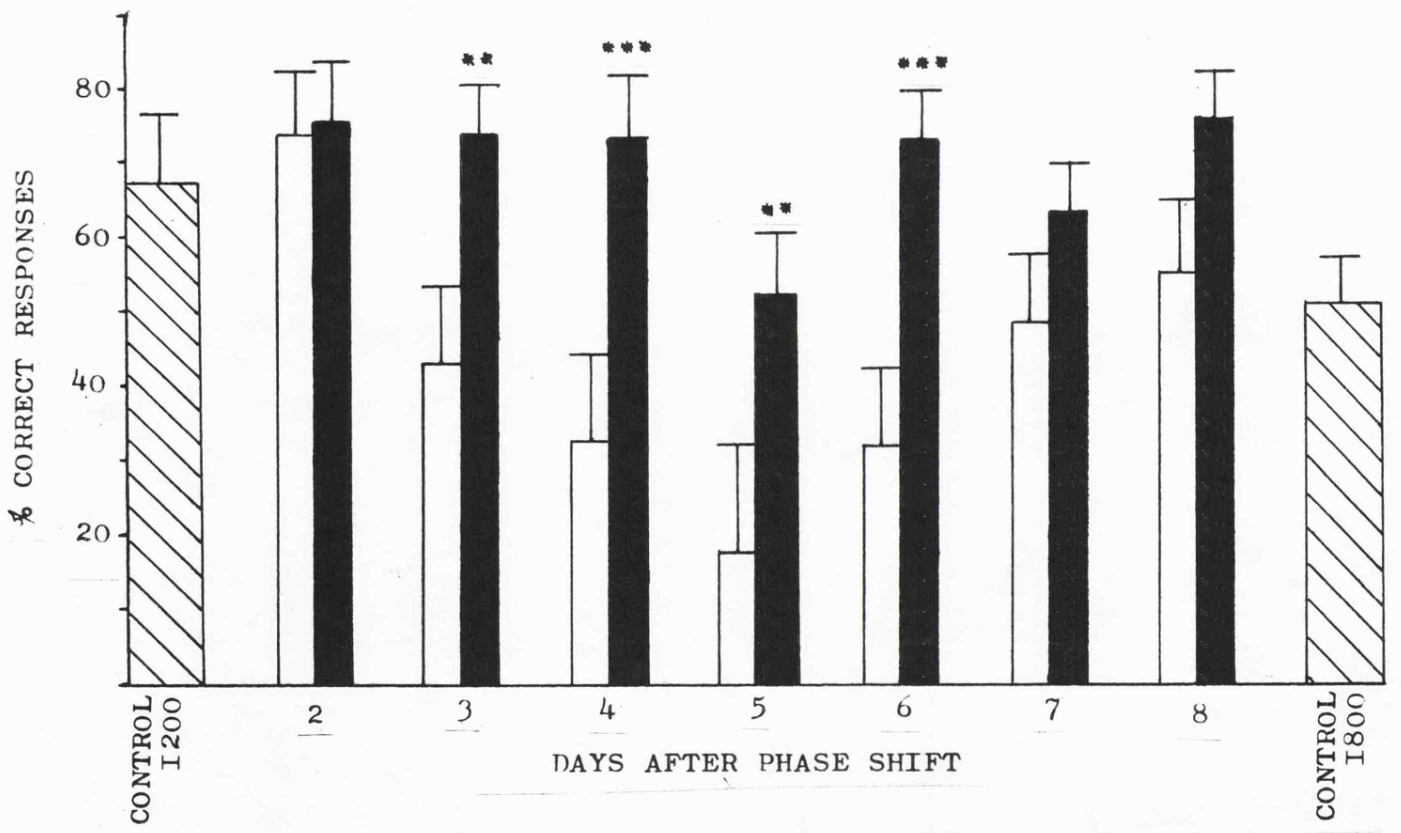


FIGURE 31 THE EFFECT ON PHASE SHIFT, OF DIFFERENT DOSES OF CHLORDIAZEPOXIDE ADMINISTERED IN THE DRINKING WATER (blank columns) COMPARED TO PHASE SHIFTED CONTROLS (hatched columns). NON-PHASE SHIFTED GROUPS RECEIVING DRUG ARE ALSO SHOWN (shaded columns)
TESTED I200. n=I2-20 ±S.E. *** Differs p<·002, ** p<·02

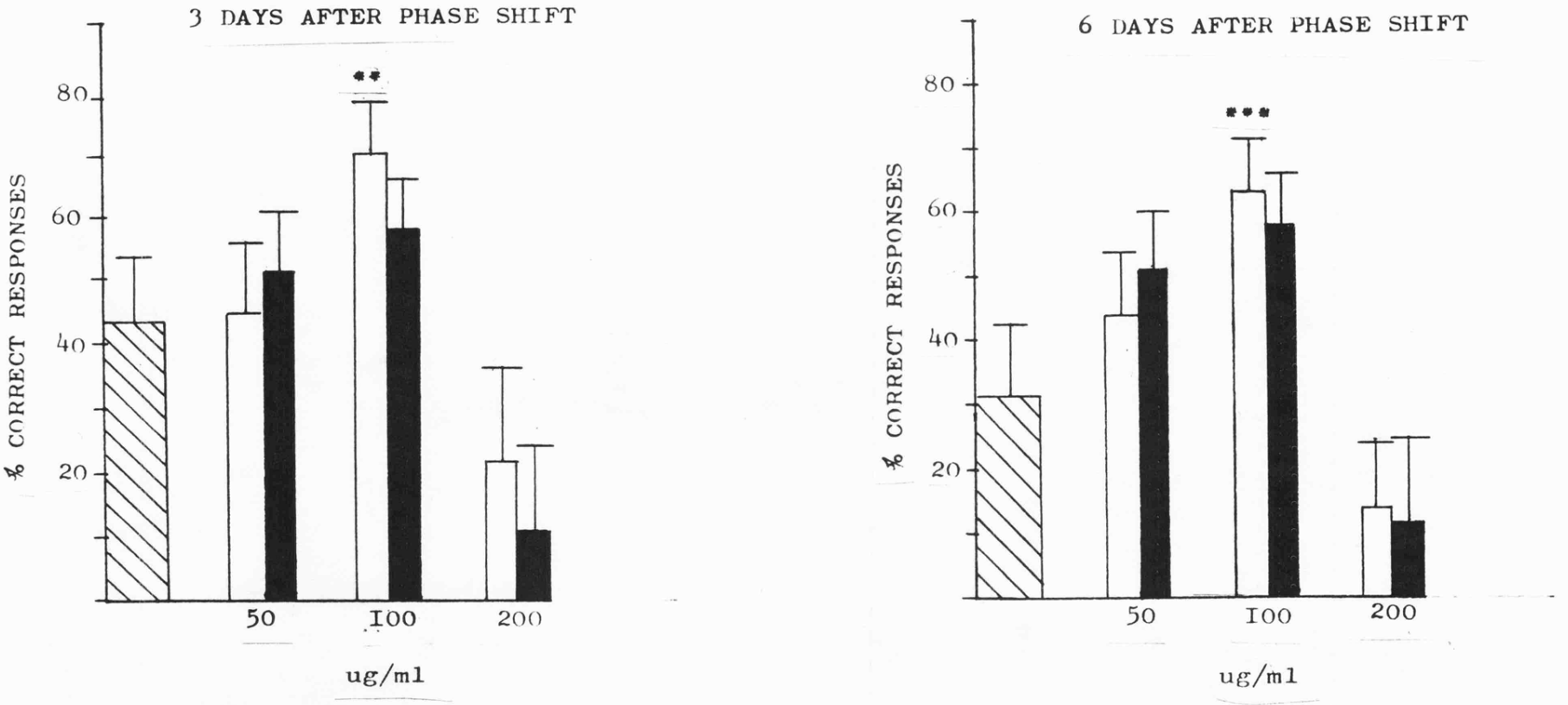


FIGURE 32 THE EFFECT OF CHLORDIAZEPOXIDE INJECTION ON PHASE SHIFT (shaded columns) COMPARED TO PHASE SHIFTED CONTROLS RECEIVING SALINE (blank columns) AT TWO CLOCK HOURS. NON- PHASE SHIFTED CONTROLS RECEIVING SALINE ARE ALSO SHOWN (hatched columns) n=II-I2 ±S.E. *** Differs from non drug treated, phase shifted, p<·002, ** p<·02, * p<·05

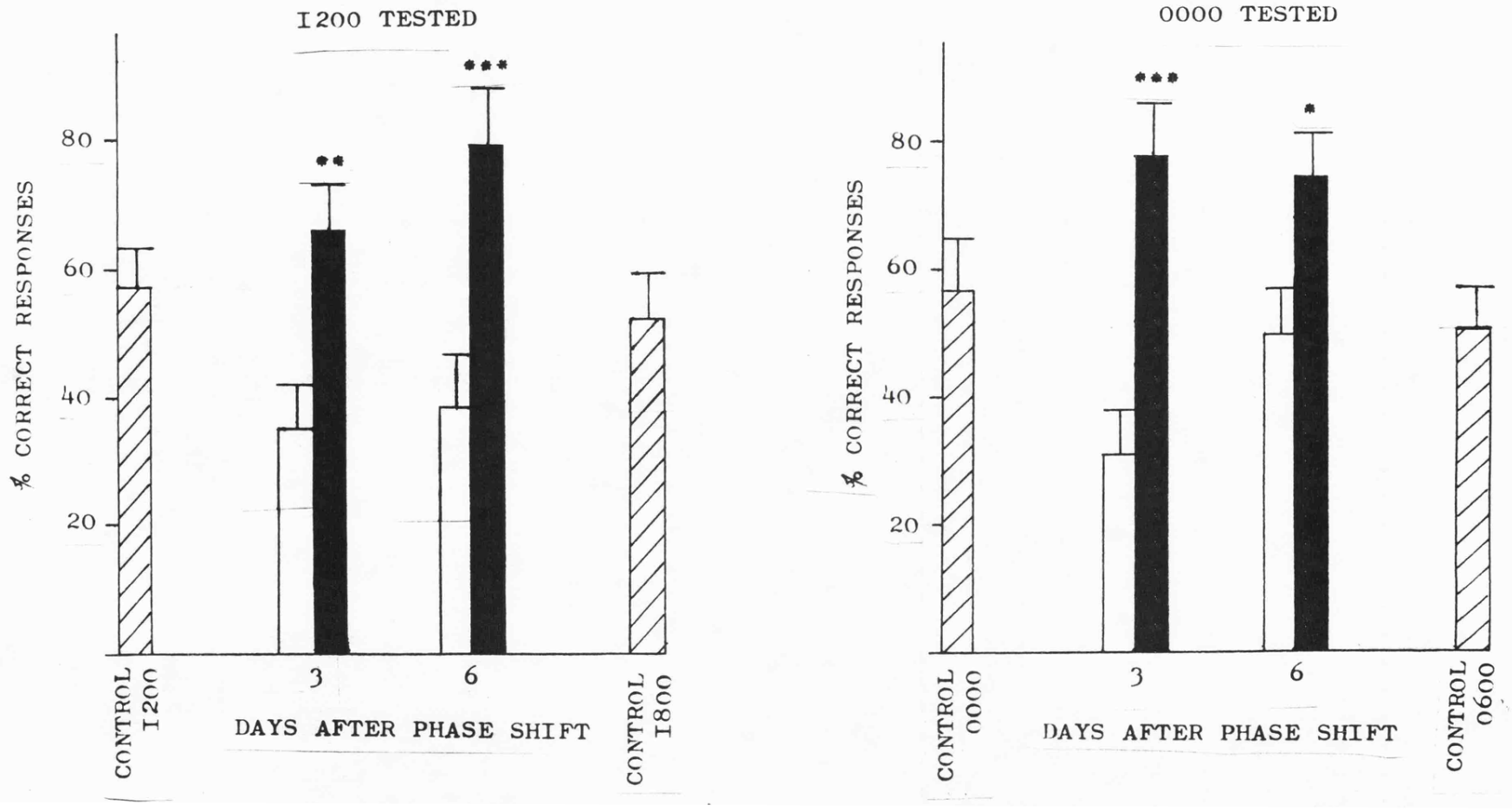


FIGURE 33 THE EFFECT OF CLOBAZAM INJECTION ON PHASE SHIFT (shaded columns) COMPARED TO PHASE SHIFTED CONTROLS RECEIVING SALINE (blank columns) AT TWO CLOCK HOURS. NON-PHASE SHIFTED CONTROLS RECEIVING SALINE ARE ALSO SHOWN (hatched columns)

n=I2-I3 ±S.E. *** Differs from non-drug treated, phase shifted, $p < .002$

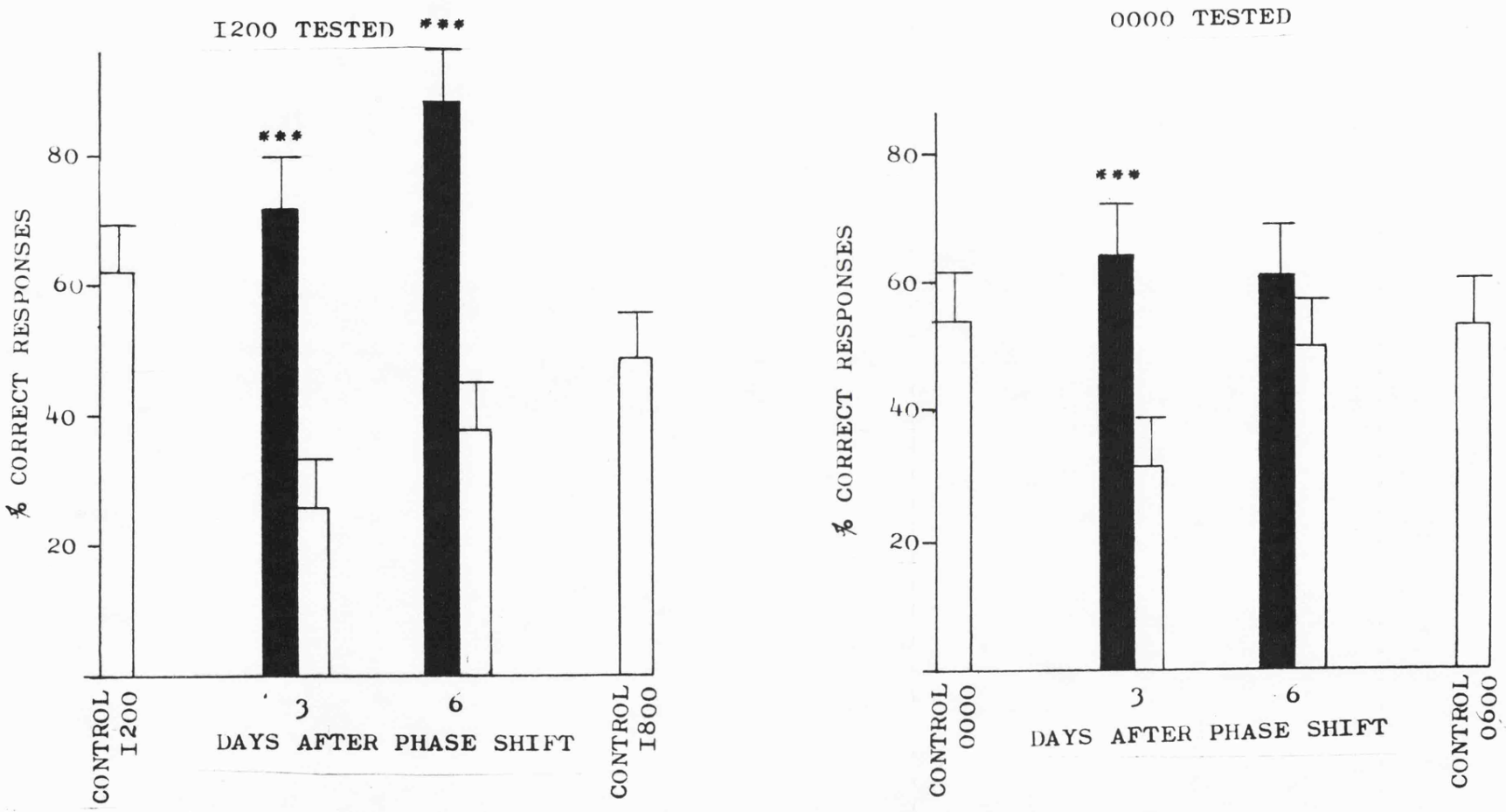


FIGURE 34 THE EFFECT OF DIAZEPAM INJECTION ON PHASE SHIFT(shaded columns) COMPARED TO PHASE SHIFTED CONTROLS RECEIVING SALINE (blank columns) AT TWO CLOCK HOURS. NON-PHASE SHIFTED CONTROLS RECEIVING SALINE ARE ALSO SHOWN.(hatched columns).
n=10-14 ±S.E. *** Differs from non drug treated, phase shifted, $p < .002$

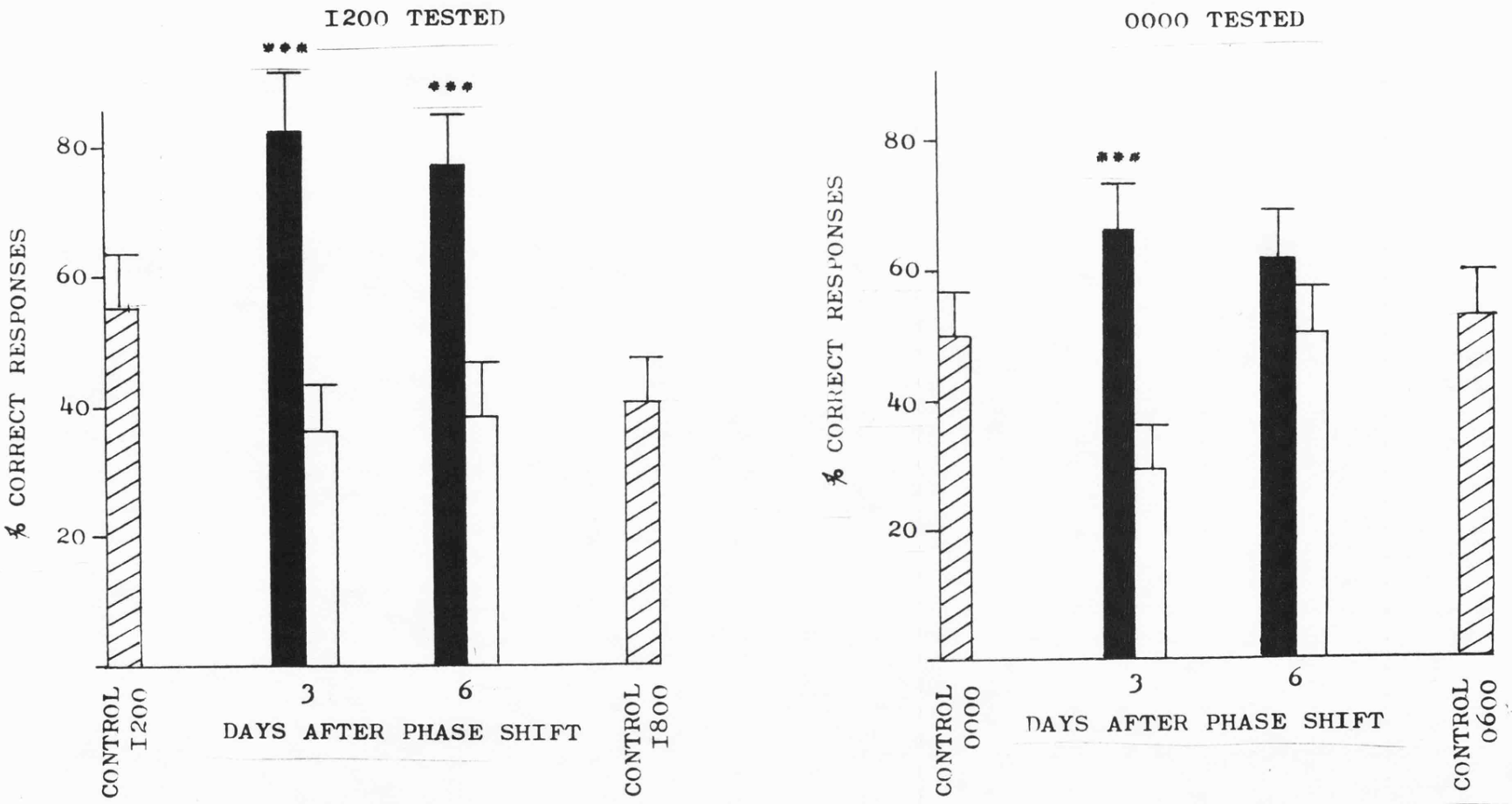
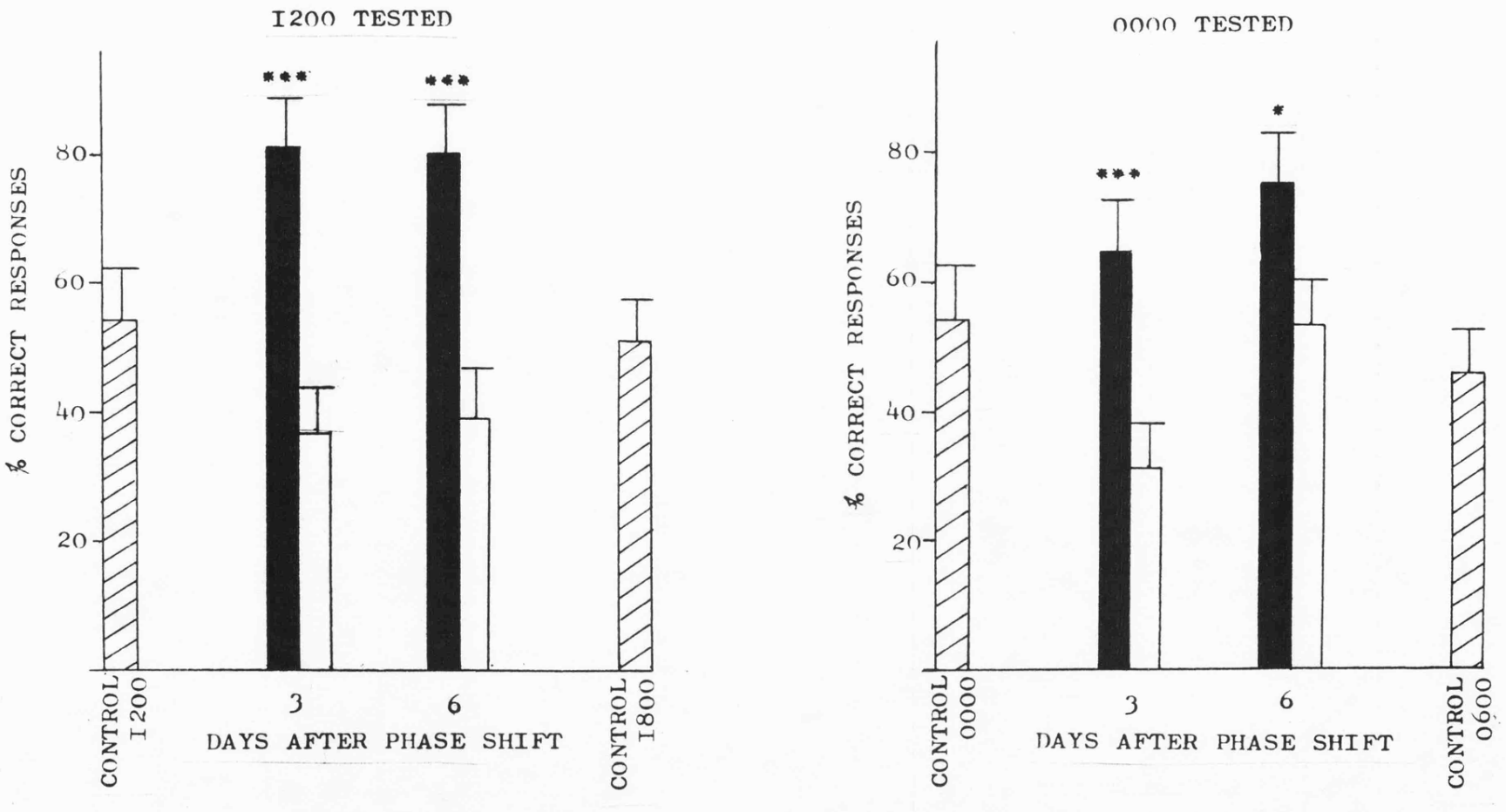


FIGURE 35 THE EFFECT OF TEMAZEPAM INJECTION ON PHASE SHIFT (shaded columns) COMPARED TO PHASE SHIFTED CONTROLS RECEIVING SALINE (blank columns) AT TWO CLOCK HOURS. NON-PHASE SHIFTED CONTROLS RECEIVING SALINE ARE ALSO SHOWN (hatched columns)

n=I2 ±S.E. *** Differs from non-drug treated, phase shifted, p<·002, * p<·05



otherwise stated) following the shift, at the animals' dark onset, and were statistically compared with the I800 non-phase shifted control group (see fig. 26) which constituted the new "expected level" of response. Results are represented as histograms, c.f. Davies, et al, (I974), and acknowledgement is given to these authors, in that their method of expressing their results has been adopted in this case.

Thus, as reference to fig. 25 illustrates, a group of animals phase-advanced by 6 hours at I200 receives I8 hours of dark in the subsequent 24 hours. Conversely, a similar phase shift at 0000 hours results in I8 hours of light in the subsequent 24 hour cycle.

The effect of a single 6-hour advance was assessed by measuring PAR in different groups of mice at I200 hours local time, for 8 days after phase shift. It must be noted that I200 hours local time now corresponds to I800 hours biological time, and daily samples are compared to I800 controls (non-phase shifted animals). For example, see fig. 26. It can be seen from fig. 26 that the effect of phase shift is to reduce the level of PAR significantly, an effect which achieves its maximum on day 5, and which is consistent with an entrained rhythm resynchronizing to altered time cues. The period of maximum disturbance (day 5) also coincides with high individual variance (reflected in the high standard error). Interestingly this higher variance is not noted in animals when monitored at 0000 hours (fig. 27), and this time compared to controls at 0600. A "stepped" profile is again noted, with maximum reduction on day 4, and apparent resynchronization at day 7.

As phase shift induces a significantly lower magnitude of response than is observed under normal conditions at any time over 24 hours (fig. I2), it is concluded that phase shift has the effect

of reducing / disrupting the passive avoidance response, and probably results in the overall damping of the 24-hour rhythm for this activity, though more frequent monitoring at 2 or 4 hour intervals would need to be carried out in order to conclusively demonstrate the total disruption of the 24-hour rhythm.

Mathematical models of oscillatory systems have shown (e.g. Aschoff, I960) that it is easier to develop a new 24-hour cycle by increasing the period length of the original cycle until new and old rhythm patterns become superimposed upon one another. A phase advance would have the effect of lengthening the period, therefore facilitating resynchronization, as opposed to a phase delay which would increase the mean light intensity (I8 hours of light) and shorten the period length. Thus, in accordance with Aschoff's rule the phase advance should facilitate quicker resynchronization in dark-active species, and , it is hypothesized, would result in a longer resynchronization period following a delay in this species.

6.2 The effect of the benzodiazepines on phase shift

The use of drugs to offset the adverse effects of phase shift has been reported. For example, Ehrenstein, et al (I972) used oxazepam in an attempt to alleviate abnormal sleep patterns in shift-working nurses, by lengthening the day-time sleep period. Similarly it has been reported that airline pilots use sedatives (sleeping tablets) after rapid translocation to help them sleep (Preston & Bateman, I970).

The only published work on drug-effects on phase-shifted animals appears to be that of Davies, et al, (I974). These workers reported a dose-dependent lessening of the disruptive effects of phase shift in rats, and a more rapid adaptation to the new LD cycle, when chlordiazepoxide was administered in the drinking water.

It was decided in the fist instance, to adhere to the method adopted by these workers, to avoid the experimental difficulties associated with injection, outlined in the previous chapter. Since benzodiazepines are light-sensitive in solution (Roche literature), drugs were administered via water bottles protected from the light with aluminium foil. Drugs employed were chlordiazepoxide (I00ug/ml), medazepam (25ug/ml) and clobazam (I5ug/ml), i.e. those doses previously found to provide strong facilitation. Long-acting benzodiazepines were chosen, as the shorter-acting counterparts do not appear to facilitate passive avoidance to the same extent (e.g temazepam, fig.23).

Drugs were administered immediately before phase-shift took place, and maintained in the drinking water throughout the duration of sampling, i.e. until the eighth day after shift. As pointed out in the previous chapter, the associated disadvantage of this method is the inaccuracy in determining the dose administered and the assumption of inconsistency of ingestion of drugs throughout the day, and accumulation throughout the 8-day period.

Figs. 28, 29 and 30 show the results obtained when I00ug/ml chlordiazepoxide, 25ug/ml medazepam and I5ug/ml clobazam respectively, were administered to phase shifted animals. Results are compared to phase shifted animals receiving no drug. It can be seen that the presence of benzodiazepines in the drinking water appear to significantly reduce the disruptive effect of phase shift, and also to reduce the high individual variance. Both chlordizepoxide and clobazam abolished the "stepped" profile, and induced a smoother transition and resynchronization to the new light cycle (c.f. Davies, et al, I974).

25ug/ml medazepam (fig. 29) shows a less pronounced

facilitation of response in phase-shifted animals and does not affect the stepped profile. This may reflect the finding that medazepam is less satisfactory than the other long-acting drugs in facilitating passive avoidance (see fig. 2I).

6.3 Effect of different doses of chlordiazepoxide administered in the drinking water

Immediately preceding phase shift, different groups of mice were subjected to various doses of chlordiazepoxide dissolved in the drinking water, with treatment continuing throughout the duration of sampling, which took place twice, at three -day intervals. This experiment was designed to reveal possible variables associated with different drug dosage, and to confirm the previous study which indicated that IOOug/ml produced the greatest facilitation.

Fig. 3I compares the response obtained on the third and sixth day after shift, at doses of 50, IOO and 200ug/ml, to phase shifted controls from a previous experiment.

The results confirm IOOug/ml to give the strongest facilitatory effect at both 3 and 6 days following phase shift, with some sedation noted at 200ug/ml. It also appears that deployment of drug in the drinking water, produces a similar drug-response to that of i.p injected subjects.

6.4 Effect of drug injection on phase shift

A further series of experiments were initiated, involving drug administration by i.p. injection, half-an-hour preceding each trial. A comparison with drug administration via the drinking water was thought to be important, as a previous study had indicated that the method of drug-treatment could possibly influence results. It was also of interest to ascertain whether optimal doses

found for non-phase-shifted subjects also applied in the case of phase-shifted animals. Also a cross-check could be carried out by comparing these phase shifted, drug injected animals (i.e those receiving a known quantity of drug) with phase-shifted counterparts receiving the drug via the drinking water (fig. 3I), as, for the reasons discussed previously, these animals may be subject to inconsistency in drug plasma levels which may in turn, render re-synchronization profiles unreliable. Figs. 32 and 33 reveal the response to phase shift in animals treated with 5mg/kg chlordiazepoxide and 2.5mg/kg clobazam respectively, monitored at both I200 and 0000 hours. The results suggest these drugs to behave in a similar way in that they both modify the impairment of PAR in phase shifted animals, and that this process takes place regardless of the route of administration (re fig. 3I). It may be tentatively concluded from this result that the assumed unreliability in dosage, when administered in the drinking water, does not exist to the extent that the results may be significantly altered.

Figs. 32 and 33 also show the facilitatory effect of 5mg/kg chlordiazepoxide and 2.5mg/kg clobazam, when different groups of animals are monitored at 0000 hours. This may indicate that time-of-day effects may not constitute a significant variable for phase shift, or for drug-response to phase shift. Comparable facilitation in phase-shifted mice may be noted with 2.5 mg/kg diazepam (fig.34) at both clock hours tested.

5mg/kg temazepam (fig. 35) also elicits significant facilitation at both I200 and 0000 hours. This is unexpected as the drug was previously shown (fig. 23) to induce little measurable effect in non-phase-shifted animals.

FIGURE 36 EFFECT OF RETRIAL INTERVAL ON THE PASSIVE AVOIDANCE RESPONSE.
n=12-14 ±S.E. *** Differs from 24 hours p< ·002; ** p< ·02

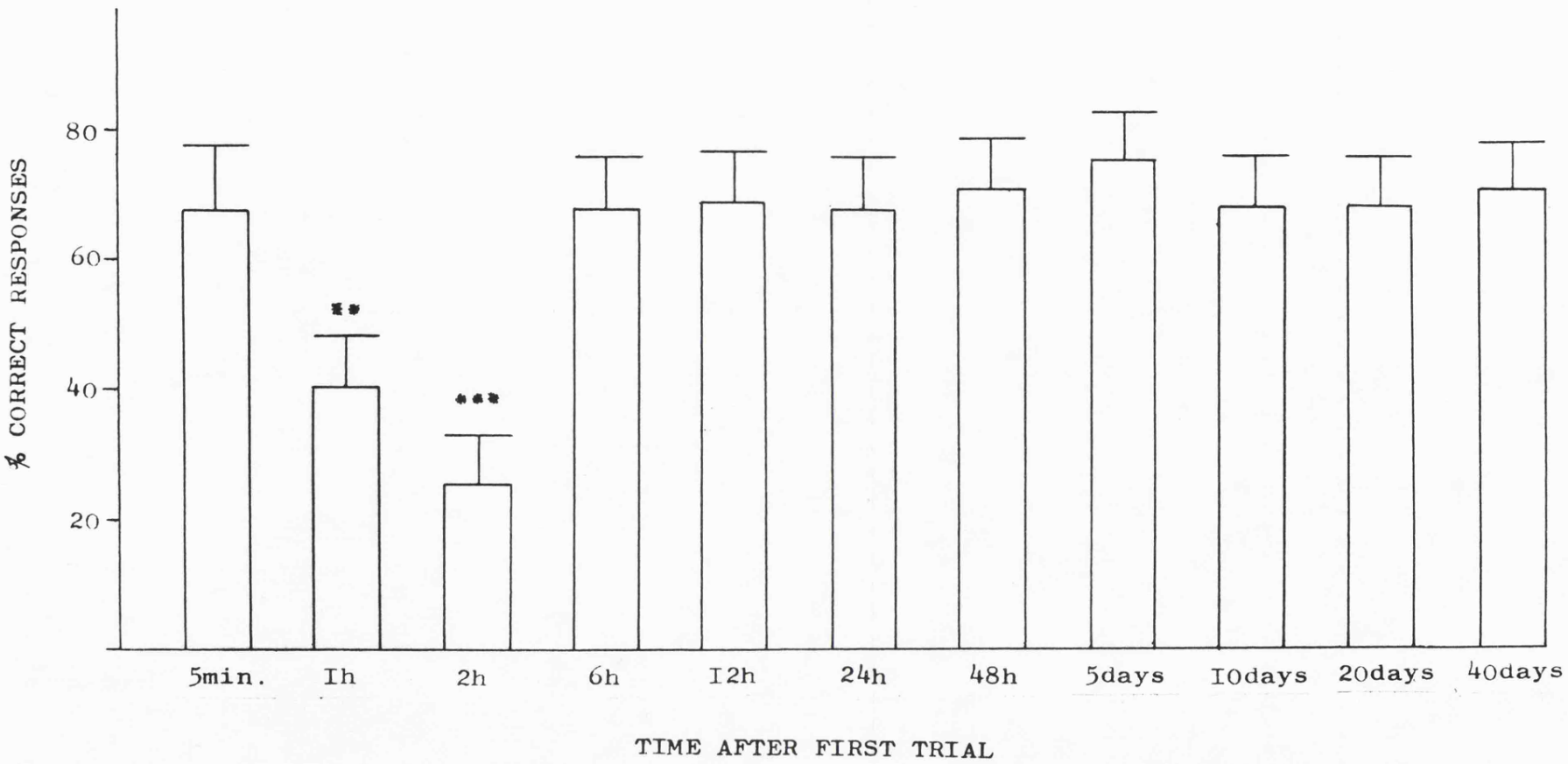


FIGURE 37 EFFECT OF RETRIAL INTERVAL ON THE PASSIVE AVOIDANCE RESPONSE & THE EFFECT OF CHLORDIAZEPOXIDE (SHADED COLUMNS). n=II-I2 ±S.E. COMPARISONS WITH SALINE CONTROLS (blank columns)

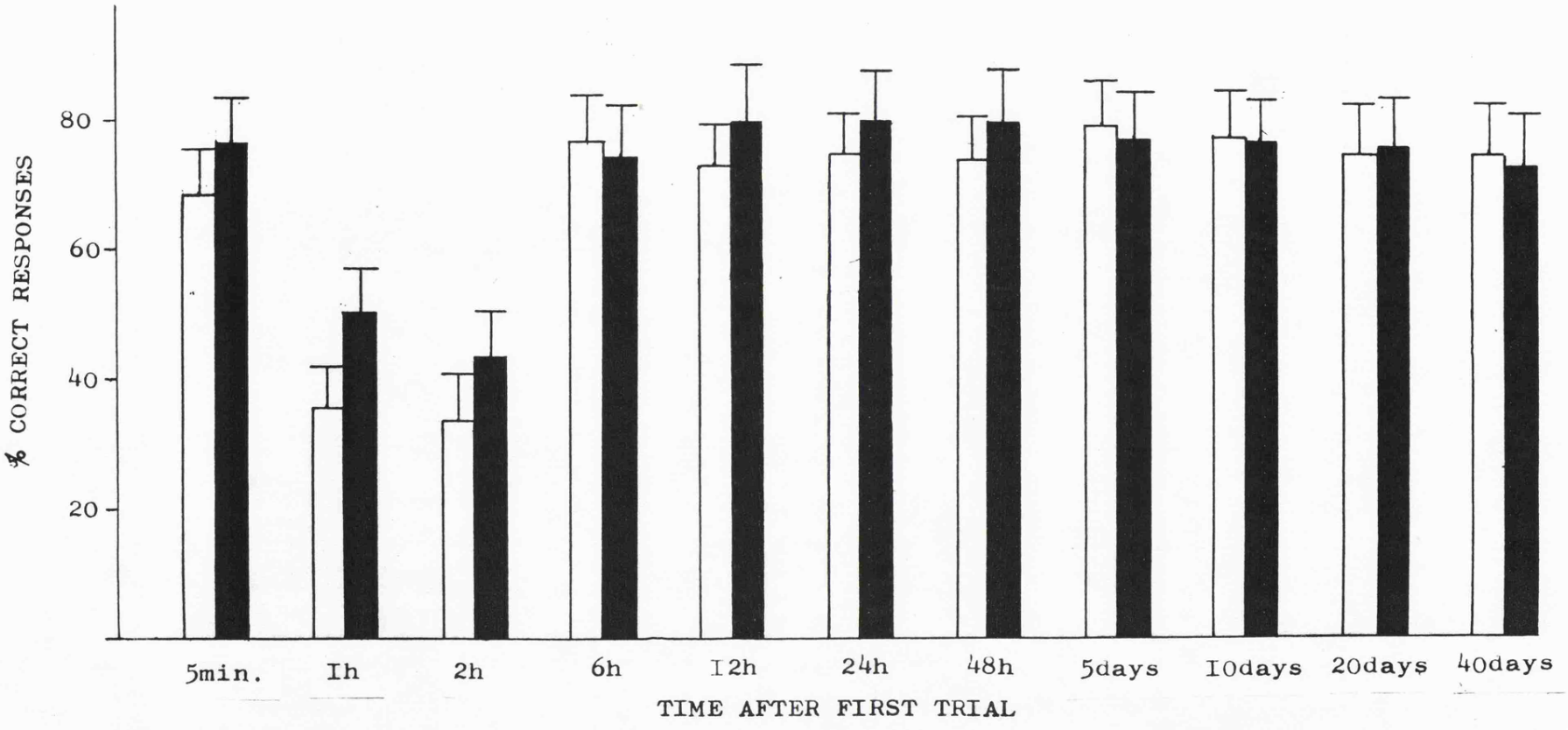


FIGURE 38 EFFECT OF PHASE-SHIFT (HATCHED COLUMNS) ON THE PASSIVE AVOIDANCE RESPONSE WITH VARIABLE RETRIAL INTERVALS. TESTED MIDDAY: n=II-I3 ±S.E. *** Differs from non-phase-shifted (blank columns) p<·002.

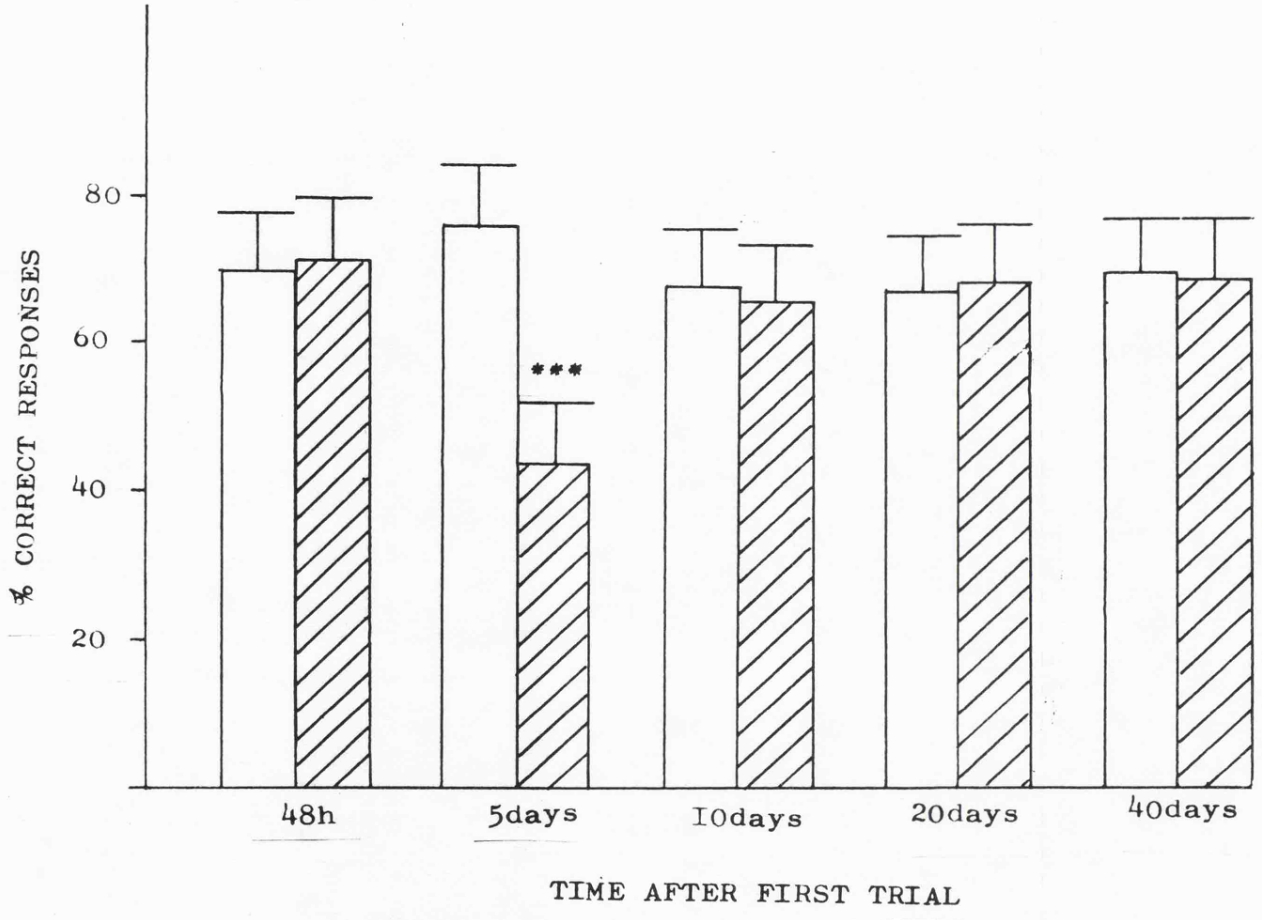
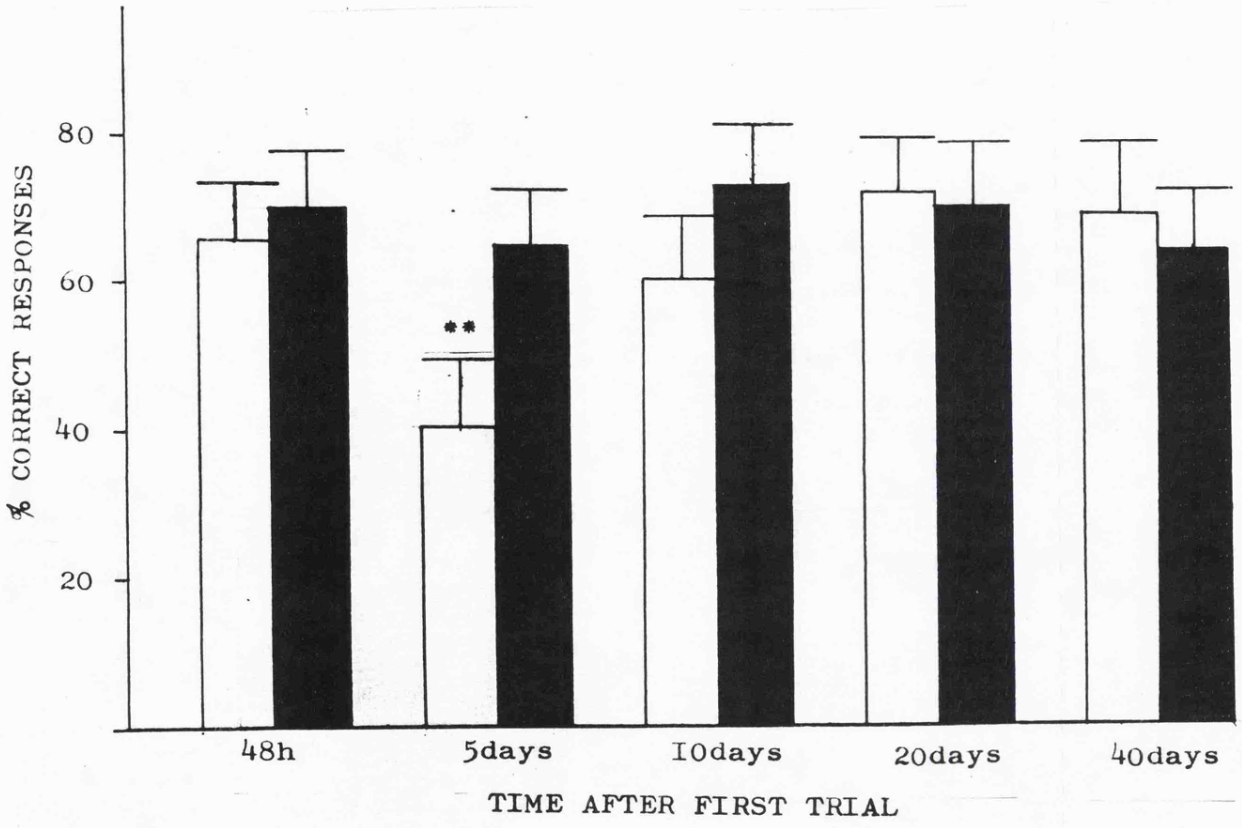


FIGURE 39 **EFFECT** OF PHASE-SHIFT WITH VARIABLE RETRIAL INTERVALS ON THE PASSIVE AVOIDANCE RESPONSE AND THE EFFECT OF CHLORDIAZEPOXIDE. (SHADED COLUMNS).

TESTED MIDDAY. n=I2-I3 ±S.E. ** Differs from saline-treated, phase-shifted (blank columns) p<·02;



6.5 Passive avoidance and memory formation

A detailed examination was undertaken to further examine recall variables associated with passive avoidance, with particular interest directed to possible short and long term memory effects. The basic methodology involved variable retrial intervals whilst maitaining the acquisition trial as a constant. Consequently, groups of I2 mice were trained at I200 hours (designated zero time), and were given retrials following variable intervals of I,2,6,I2,24 and 48 hours, and 5,I0,20 and 40 days.

Fig. 36 illustrates the response to these manipulations, which show subjects to give a mean score of 67.3% correct responses 5 mins. after training. However mean scores fell to 26.4% after 2 hours of retrial interval and consequently rose to a scoreline of 65-75% correct responses at the 6-hour interval, persisting for 40 days at this level.

The low response at the one and two-hour intervals are possibly attributable to the stressful effects of the immediately preceding trial. It is also conceded that these first four intervening retrial periods do not take account of the "Kamin effect" which has been shown to result in multi-phasic retention deficits at periodic retrial intervals of other than I2-hour multiples, in avoidance learning (Holloway & Wansley, I973)

However comparable effects have been noted with rats (Navaratnam, I973), and it seems a more likely explanation that this short-term deficit results from a transitionary stage involving extinction of short-term memory and the incompletion of a long-term memory "trace" (c.f. Navaratnam, I973). The persistence of the response for up to 40 days after training is surprising, and one may surmise, may parallel the reported skill of older, wild rodents, in

avoiding predators, trapping etc. as a result of their "long memory" (Calhoun, I962).

A second experiment was then carried out to determine the effect of benzodiazepine administration (5mg/kg chlordiazepoxide injected i.p. 30 mins. before each trial) on these variable retrial intervals compared to animals receiving saline (fig. 37) The drug apparently induced a small but not significant increase in response at I and 2 hour intervals, i.e. the hypothetical period of long-term memory consolidation. These results are insufficient to enable any firm conclusions to be drawn, other than benzodiazepine treatment does not appear to differentially influence short or long-term recall.

As all subjects were injected prior to both first and second trial (30 mins. before where possible) it must be noted that this would have the effect of substantially raising the plasma levels of drug of those groups subjected to the shorter retrial periods, with respect to those receiving long retrial periods. In the interests of inter-group comparability however, it was thought to be important to remain consistent with pre-trial injections, though it is conceded that this may have led to an additional dosage variable.

In the next experiment the effect of phase shift on different retrial intervals was examined. The effect of a 6-hour advance (instituted immediately after training) on retrial intervals of 48h, 5 days, I0, 20and 40 days is illustrated in fig. 38. The results reveal a lowered response on the fifth day after training which is not substantially different from phase-shifted subjects trained 24 hours previously (fig. 26). It thus appears that the timing of the acquisition trial is unimportant in achieving the

phase shift-induced reduction in PAR and that the second (recall) trial may more fully reflect the effects of phase shift. Recall of the response was fully restored by the IOth day after shift and no long-lasting vestigial effects of phase shift may be noted.

Finally chlordiazepoxide was administered i.p. to phase shifted animals, preceding both acquisition and recall trials for a period of up to 40 days, in an attempt to discover whether drug treatment during the first few days had simply delayed the disruptive effects of phase shift, or had in fact alleviated the disruption. The results are illustrated in fig. 39 and appear to show that the reduction of PAR was not merely delayed, but prevented altogether.

6.6 Discussion

The results reported in this chapter show that a 6-hour phase advance results in a reduction of the passive avoidance response before attainment of relative stability and the new "steady state", when monitored at both I200 and 0000 hours. A critical stage appears to be reached at around the 4th or 5th day after shift, when the lowest response occurs, after which resynchronization apparently takes place. Navaratnam (I973) has reported that phase shifts carried out at different times, resulting in irregular periods of light or dark, were instrumental in determining the amount of disruption which occurred. Thus it would appear important to undertake future experiments involving phase shifts carried out at different times, to compare the effects of the resultant changes in mean light intensity, and to see if they conformed to the "Aschoff rule for dark-active species".

As one of the characteristics of an endogenous rhythm is its disruption following a change in external zietgebers, it

is possible that the observed reduction represents total disruption of a circadian (endogenous) rhythm in passive avoidance behaviour, though further 24-hour monitoring would need to be carried out in order to conclusively demonstrate this. However, because the PAR does not immediately coincide with changes in the light cycle, this is no reason to suppose the behaviour is not synchronized with light (see later discussion). The behaviour may be controlled indirectly by light, by an endogenous oscillator coupled to an exogenous receptor (see Aschoff, 1960) responding directly to the illumination cycle. Thus these two components may drift out of phase with each other, in turn influencing those behaviours under the control of this oscillator mechanism. Following some time-interval, this out-of-phase physiological clock may "catch up" with the new external cycle, and once more drift into phase with this exogenous component.

It has also been shown that the benzodiazepines administered both intraperitoneally and in the drinking water, caused a dose-dependent in PAR during the critical period of impairment following phase shift, and that generally those animals receiving the highest and lowest doses of drug did not show these improvements. The mechanism by which the drugs act remains a source of debate. It is possible that benzodiazepine treatment may facilitate faster rephasing of the hypothetical clock components, though no reported literature exists on the ability of the benzodiazepines to do this.

Perhaps the strongest possibility is that of recall facilitation by the drugs, by some mechanism or other. However other possibilities to be isolated are behavioural and peripheral action indirectly leading to appropriate responses in the passive avoidance situation. The anxiolytic properties of the drugs must also be

considered, as clearly, an anxiety state would be detrimental to learning and recall, while drug treatment may remove these learning "barriers" by their known disinhibitory properties.

Direct extrapolation from this murine model to the human condition are clearly dangerous, as parallels with translocated humans are complicated by the fact that "flight stress" has not occurred. However it seems plausible that psychological disturbances due to "flying time" coupled with desynchronization of endogenous rhythms, may both contribute to the symptoms of "jet lag" (c.f. Hale, et al, I972), and that this animal model could function as a useful parallel to the known "efficiency loss" in many psychomotor tasks performed by phase-shifted human subjects.

Chapter 7

SOME PRELIMINARY INVESTIGATIONS INTO THE EFFECTS OF OTHER CLASSES OF DRUG

7.I Introduction

In considering the effects of the benzodiazepine derivatives on passive avoidance, it seemed logical to extend this study to encompass centrally-acting drugs from some other different classes, the rationale being:

(a) To establish whether the facilitatory effects noted in the benzodiazepines are common only to this class of drug.

(b) To determine whether different classes of drug can be differentiated on the basis of the animals' time of day susceptibility to them.

(c) To determine whether these drugs can provide any facilitatory effects in phase shifted groups, or on the other hand, to ascertain any possible potentiation of the ill-effects.

The drugs chosen were selected either for their obvious clinical importance (e.g. alcohol, chlorpromazine), or were of particular interest due to other miscellaneous properties, later described. The selected drugs were as follows:

(I) Chlorpromazine

Chlorpromazine is one of the most frequently investigated therapeutic drugs, and is widely used in the treatment

of schizophrenia. It was thought to be of particular interest in the passive avoidance situation as (a) It has been shown to disrupt performance in shock-motivated tasks (Fischman & Schuster, I979), which has been explained as resulting from the drug's reduction of fear produced by footshock, and (b) the phenothiazines generally appear to be effective in suppressing arousal (Bradley, I963), and (c) the drug reduces locomotor activity (Ban, I969).

Thus these behavioural characteristics of the drug could further prove useful in the study of what actually motivates an animal to cross (or be inhibited from crossing) from plate to plate in the passive avoidance task, and whether it is in fact a valid test for learning and memory.

(2) Clomipramine

The tricyclic antidepressant drugs (TAD) show their chief therapeutic utility in the elevation of mood. Also, recent investigations have drawn attention to the possibility of such TADs as clomipramine being used in the relief of chronic pain (Budd, I978 ; Laplane, I979). For some authors the analgesic properties of these drugs are directly related to the elevation of mood (Ward, et al, I979), while others have suggested these drugs to possess specific analgaesic properties (Laplane, I979). In order to account for this, biochemical mechanisms common to both mood control and nociceptor influence have been invoked (Lee & Spencer, I977).

The molecular mechanisms underlying the therapeutic effects of tricyclic and tetracyclic antidepressants is not yet firmly established. One hypothesis concerns the possible blockade of the uptake of noradrenaline, 5-HT, or dopamine by these drugs (Messing & Lytle, I977; Ross & Renyi, I975; Kuhar, et al, I972) Korf, et al, (I978) have shown that repeated treatment with TADs

interferes with the generation of cyclic AMP by noradrenergic neurons of the locus coeruleus. Pronounced analgaesic effects have been reported in animal studies by, it is thought, the drugs' possible action on the endorphin systems (Eschalier, et al, I98I)

It seemed therefore of considerable interest to examine the effects of one TAD, in relation to the avoidance conditioning task, particularly as the possibility of reduced sensitivity to footshock and possible alleviation of fear, could possibly lead to changed behaviour in passive avoidance trials. Clomipramine seemed of particular interest in this situation, as the drug has recently been shown to cause reduction of the 24-hour mean in free-running locomotor activity rhythms in rats (Martin & Redfern, I982) while a number of recent studies have shown chronic antidepressant treatment to influence the frequency of various biological rhythms in animals (Wehr, et al, I979). Antidepressant drugs have also been reported to increase cycling between mania and depression in human patients (Wehr, et al, I977), which is consistent with a possible influence on desynchronization.

For reasons such as the latter, the interrelationship between the TADs and phase shift are particularly relevant.

(3) Atropine

It has been found that drugs which block the synaptic action of acetylcholine (such as atropine and scopolamine) can impair performance on learning tasks and memory (Blozovski, et al, I977). Because drugs which affect cholinergic transmission can also affect memory, the cholinergic theory of memory has received considerable impetus. Atropine also has the reputation of stimulating locomotor activity (Thornburg & Moore, I973; Nomura & Segawa, I978). Thus as well as providing information as to the

drug's efficacy (or lack of), manipulation of locomotor activity levels could provide information as to the role of activity levels in the acquisition success in PAR trials.

(4) Ethanol

The ingestion of alcohol before learning typically impairs this ability in both humans and animal species (Birnbaum & Parker,I977; Ryback, I973; Wallgren & Barry, I970), though low doses may enhance retention in some instances (Ryback, I973). The mechanism by which this process occurs is unclear, though there is a suggestion that ethanol amnesias may result from deficits in memory trace consolidation (Landauer, I977; Parker, et al, I974; Jones, I973).

The study of alcohol has obvious clinical importance especially in relation to phase shift, as consumption by air travellers could conceivably potentiate the ill-effects resulting from internal desynchronization.

7.2 Preparation and administration of drugs

All drugs were injected i.p. 30 mins. preceding each exposure, and were dissolved directly in distilled water, either in the pure or compound form, i.e atropine sulphate, chlorpromazine hydrochloride. All solutions were diluted to volume with distilled water, and administered at concentrations found by previous workers to be the optimal acute dosage for altering behaviour, without inducing motor impairment or other behavioural depression.

The dosage employed, together with the respective reference for such a dose are indicated overleaf:

Chlorpromazine	2.5mg/kg	Valzelli, et al(I967)
Atropine	2mg/kg	"
Clomipramine	20mg/kg	Eschalier,et al(I98I)
Ethanol	I.6g/kg	Krsiak, (I976)

The time-of-day effects of these drugs, relative to saline controls, at four clock hours, are expressed as histograms in fig. 40, 4I, 42 & 43 and show these drugs to be incapable of any facilitatory activity of PAR at any of the times tested. With the exception of I.6g/kg ethanol (fig.43) which showed little effect, the effect seems generally to reduce the response by the use of these drugs. Susceptibility to ethanol, 20mg/kg clomipramine (fig. 42) and 2.5mg/kg chlorpromazine (fig. 4I) appears slightly greater after dark onset. This makes interesting comparison with benzodiazepine derivatives, where the time of greatest drug-response appears to occur at light onset and throughout the earlier portion of the light phase. These different characteristics of these classes of drugs presumably reflect a different mode of action, and that this particular mode of action is responsible for facilitatory or depressant activity in passive avoidance success.

Atropine extinguished PAR at all times tested, and at 0600 and I800 hours, the recall response was inhibited to the extent that more plate-crossings were attempted on second trial than were attempted on first exposure. The response to 2mg/kg atropine at different times of day is illustrated in fig. 40. First-trial scores were comparable with controls in that the animals were not stimulated to cross more frequently (unlike the benzodiazepines). However the high frequency of second trial plate-crossings tends to lead to the supposition that retention is seriously impaired by the administration of this drug, as seems

FIGURE 40 TIME OF DAY EFFECTS ON THE RESPONSE TO ATROPINE
(unbroken line)
n=10-12 ±S.E. Differs from saline controls (broken line), $p \leq .002$
at all times tested

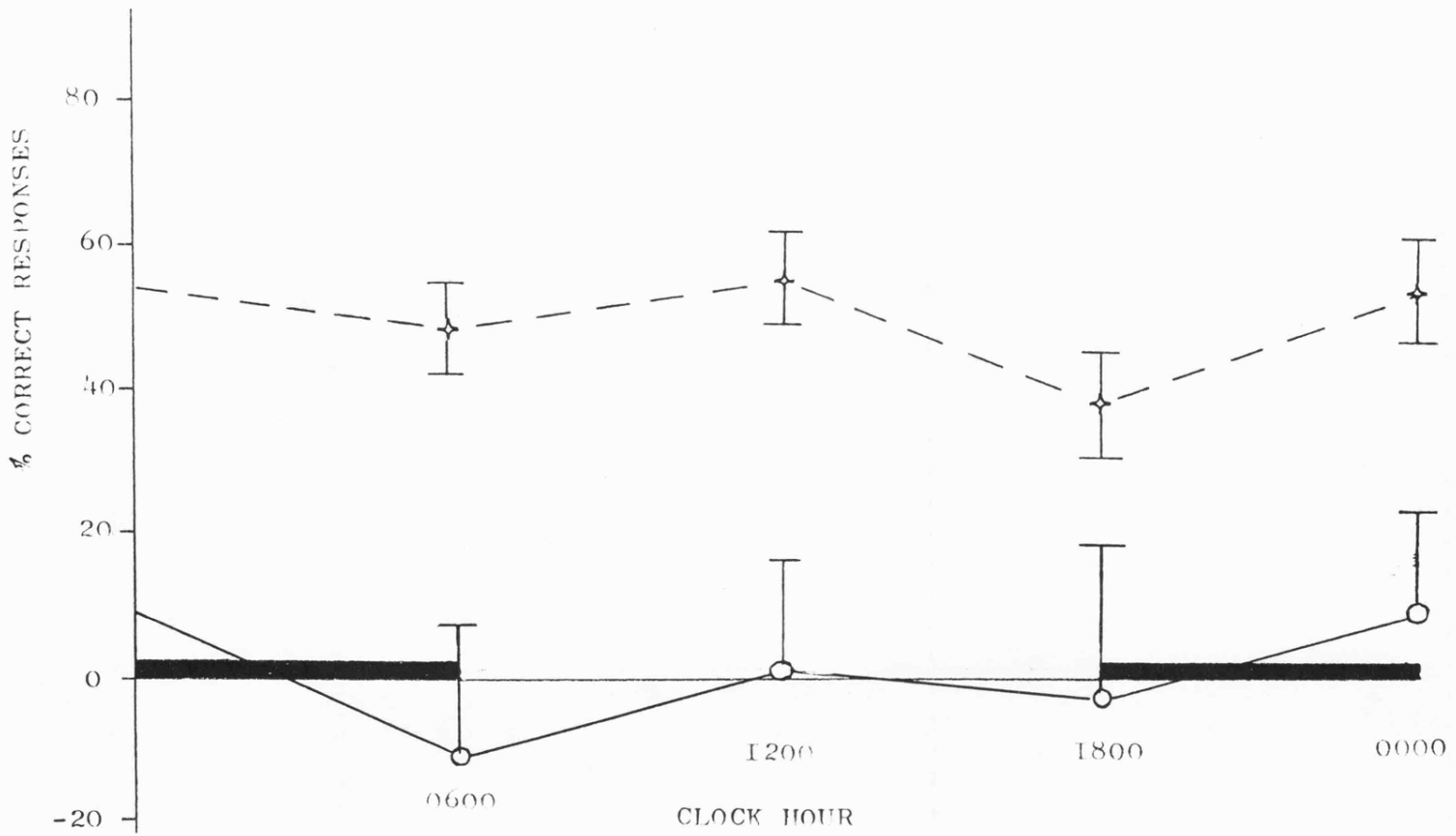


FIGURE 4I TIME OF DAY EFFECTS ON THE RESPONSE TO CHLORPROMAZINE (unbroken line)

n=10-12 ±S.E. *** Differs from saline control, p<·002, *, p<·05

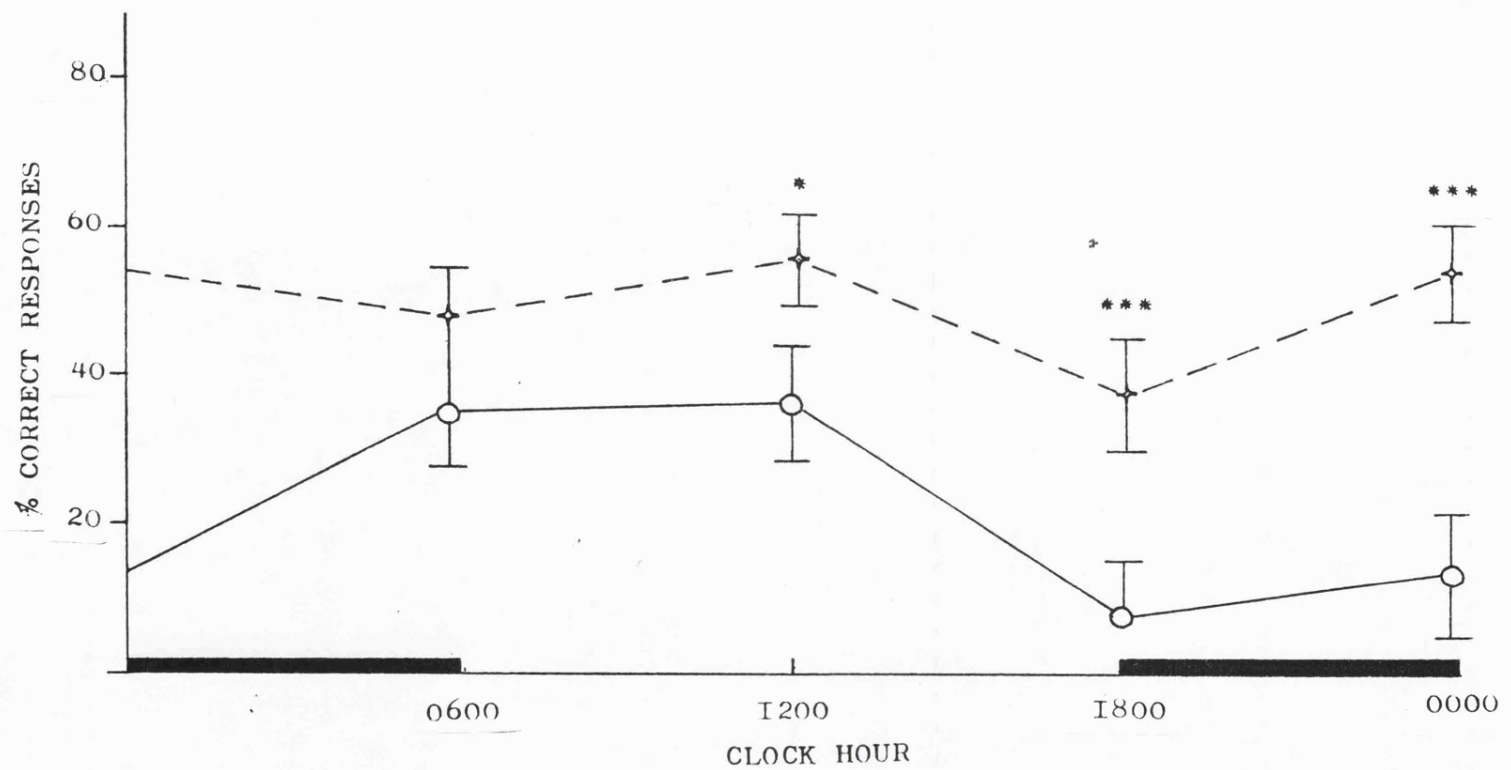


FIGURE 42 TIME OF DAY EFFECTS ON THE RESPONSE TO CLOMIPRAMINE (unbroken line)
n=10-12 ±S.E. *** Differs from saline controls (broken line), $p<.002$, ** $p<.02$

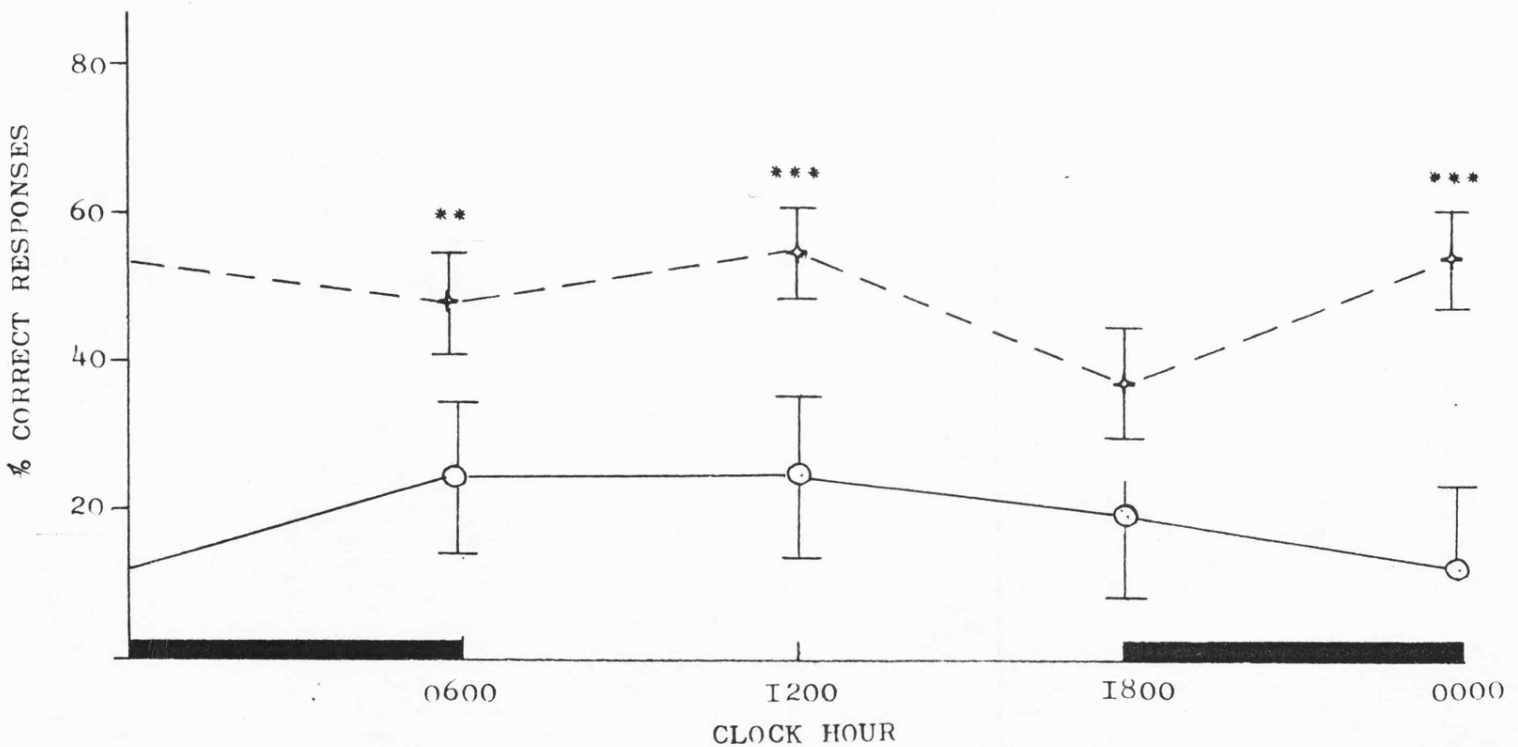


FIGURE 43 TIME OF DAY EFFECTS ON THE RESPONSE TO ETHANOL
(unbroken line)
n=10-12 ±S.E.

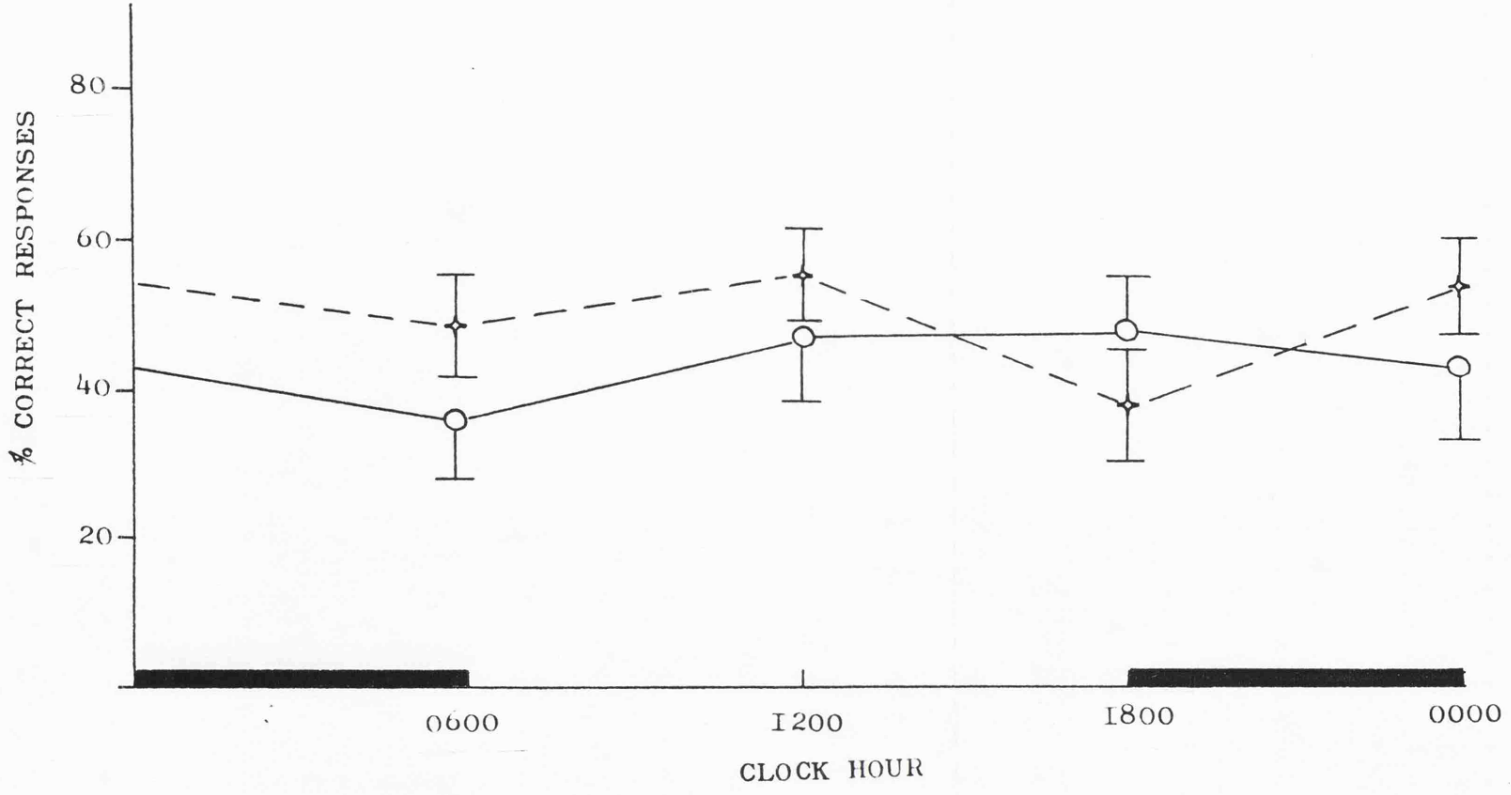


FIGURE 44 THE EFFECT OF ATROPINE INJECTION ON PHASE SHIFT (shaded columns) COMPARED TO PHASE SHIFTED CONTROLS RECEIVING SALINE (blank columns) NON-PHASE SHIFTED CONTROLS RECEIVING SALINE ARE ALSO SHOWN (hatched columns). n=10-12 ±S.E. *** Differs p≤·002

TESTED MIDDAY

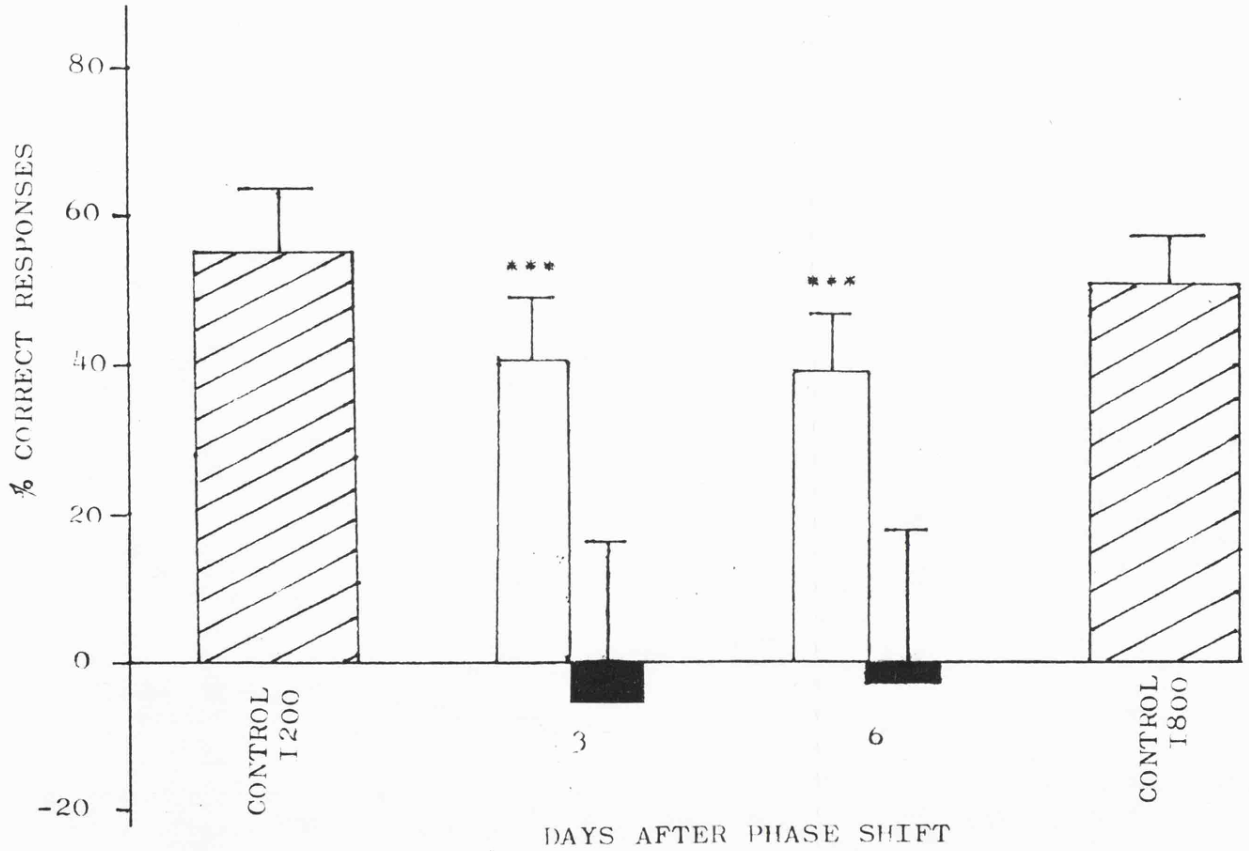


FIGURE 45 THE EFFECT OF CHLORPROMAZINE INJECTION ON PHASE SHIFT (shaded columns) COMPARED TO PHASE SHIFTED CONTROLS RECEIVING SALINE (blank columns). NON-PHASE SHIFTED CONTROLS RECEIVING SALINE ARE ALSO SHOWN.

TESTED MIDDAY n=10-12 +S.E.

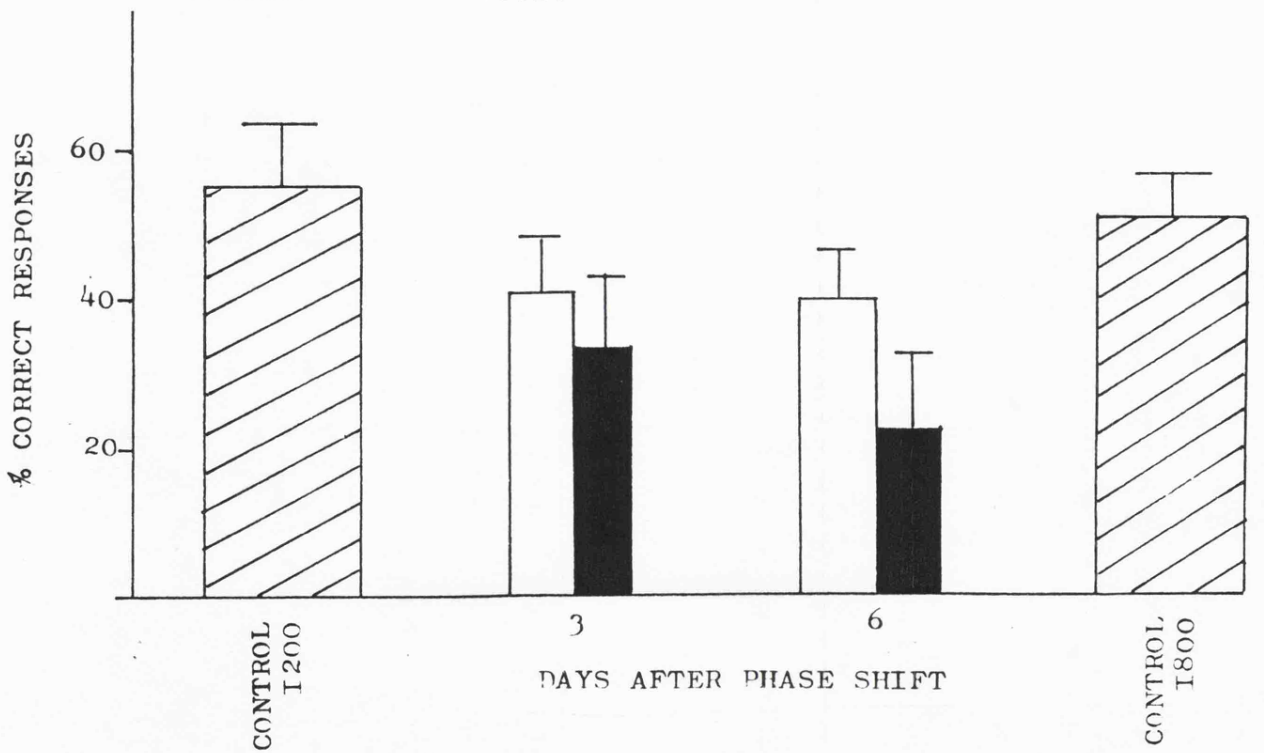


FIGURE 46 THE EFFECT OF ETHANOL INJECTION ON PHASE SHIFT (shaded columns) COMPARED TO PHASE SHIFTED CONTROLS RECEIVING SALINE (blank columns). NON-PHASE SHIFTED CONTROLS RECEIVING SALINE ARE ALSO SHOWN (hatched columns) TESTED MIDDAY n=10-12 +S.E.

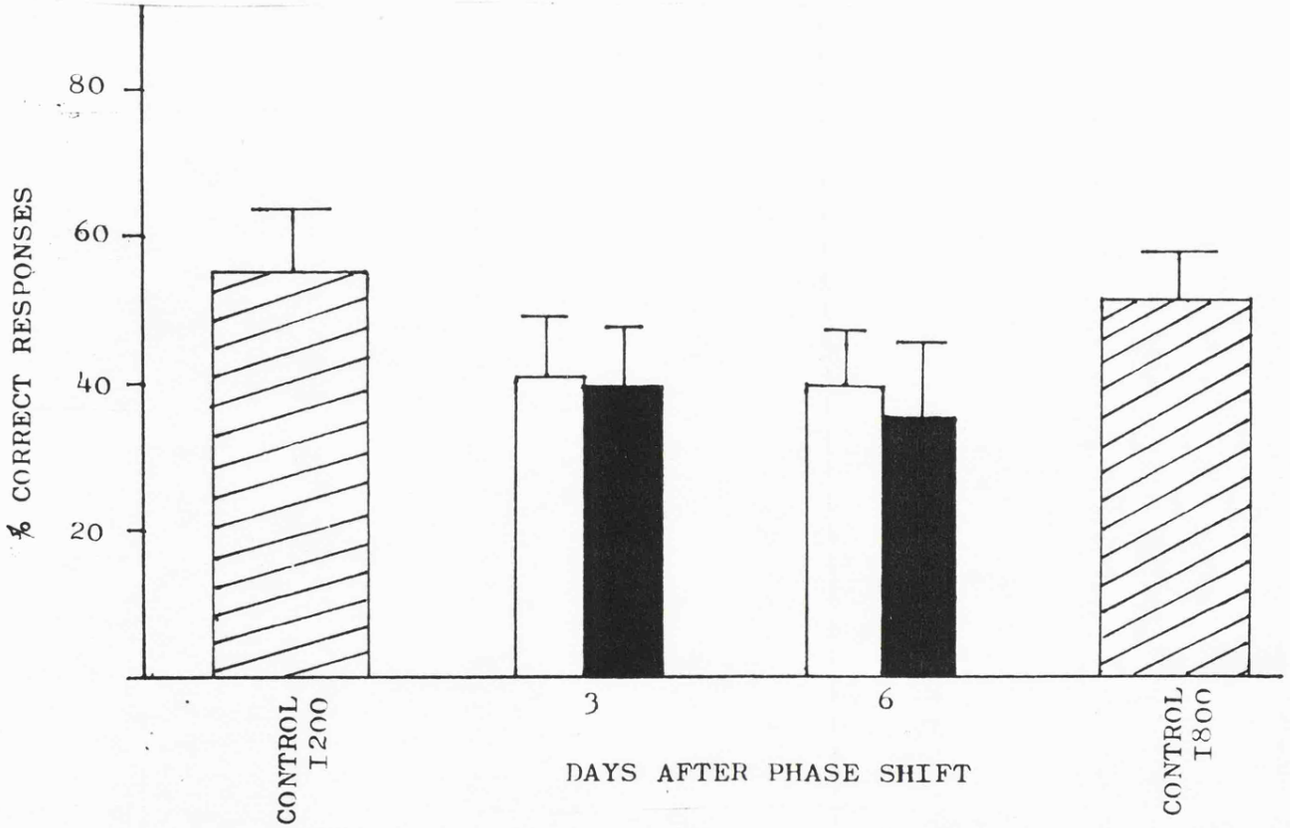
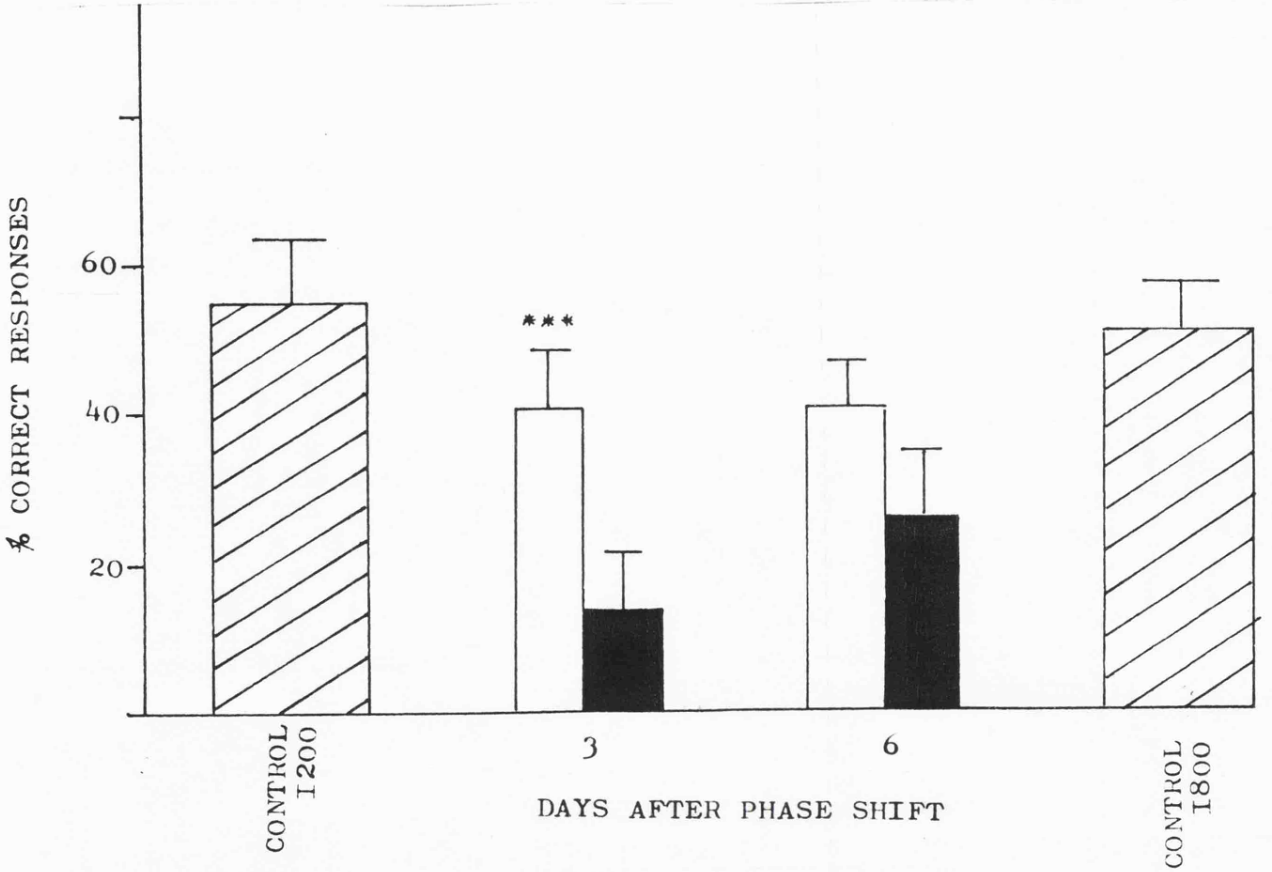


FIGURE 47 THE EFFECT OF CLOMIPRAMINE INJECTION ON PHASE SHIFT (shaded columns) COMPARED TO PHASE SHIFTED CONTROLS RECEIVING SALINE (blank columns). NON-PHASE SHIFTED CONTROLS RECEIVING SALINE ARE ALSO SHOWN (hatched columns) TESTED MIDDAY. n=10-12 ±S.E. *** Differs $p<.002$



the case with other classes of drug (though to a less extent).

Haus (I964) has stated the resistance of mice to psychoactive compounds to be at its lowest shortly after dark onset. Though apparently concurring with this observation in so far as these particular drugs are concerned, the benzodiazepines appear to be unique in that their time of maximum effect is at the opposite end of the 24-hour cycle.

7.3 Effect of phase shift

The effect of a 6-hour advance, measured at midday (local time) on the third and sixth day after shift, is examined with respect to the effect of these drugs, and illustrated in figs. 44, 45, 46 & 47. The doses employed are those of the previous experiment. None of these drugs employed in this experiment were able to provide any facilitation in phase shifted animals, a result which had not been entirely unexpected, judging by their time-of-day effects. The drugs provided a further impairment of PAR in phase shifted animals, as was the case in control groups at different times of day, thus drug treatment provided a PAR reduction which would have been expected. However the difference between phase shifted, drug-treated and non drug-treated categories was not sufficiently great to lead to the conclusion that any of these drugs had actually potentiated the disturbance induced by phase shift.

7.4 Discussion

A small sample from different drug classifications were unable to produce the facilitatory effects noted with the benzodiazepines, an effect which so far appears specific to the latter class of drugs. The effect of the benzodiazepines in facilitating the response seems most likely a result of direct facilitation of learning/memory processes by such mechanisms as

hippocampal activity, or possible influence on other brain areas. The employment of this further drug study has however provided important further information in that:

(a) Atropine, though possessing the reputation of increasing locomotor activity, did not increase plate-crossings at first trial as the benzodiazepines did (which also have the reputation of increasing locomotor activity). Due to this inconsistency, it seems logical to speculate that locomtor activity levels do not predetermine the frequency of plate-crossings at first trial.

(b) The extinction of PAR by atropine further reinforces the idea that this passive avoidance test does constitute a valid measurement of learning or retention, which is independent of activity levels . The abolition of the response by atropine is also consistent with a drug which affects cholinergic transmission and consequently adverse effects on memory.

(c) The reputed effects of chlorpromazine on animals are reduction of locomotor activity and reduced shock-motivated learning. The drug clearly did not reduce plate-crossings by this presumed inhibition of locomotor activity.

Thus the effects of these drugs on passive avoidance are useful as a means of validating this test as reflecting learning and memory recall, while locomotor activity variables appear to be irrelevant in their determination of plate-crossing frequency.

As many components of brain function, such as neurotransmitter levels, display circadian variations. It follows that centrally-acting drugs which interact with neurotransmitter function will also be subject to circadian variations in terms of

the behaviour which results. This appears especially true of mood-altering drugs, which depend on their action, on influencing synaptic uptake, turnover and metabolism. Thus the magnitude of a particular behavioural response will depend on the state of the brain at a particular time of day.

Unlike the benzodiazepines which tended to exert their greatest effect in the early light phase, the general effect of such drugs as chlorpromazine and clomipramine is increased susceptibility following dark onset, c.f. Davies' (I97I) observation with amphetamine and hallucinogens. It therefore seems probable that this reflects some specificity of action of the benzodiazepines, which a large number of other drugs apparently do not possess.

Although treatment with these drugs tended to reduce PAR in phase shifted groups, the reduction was no more than would have been expected, when considering that these drugs do apparently depress the response in non-phase-shifted groups anyway. Thus it seems that the reported property of such drugs as clomipramine, of manipulating circadian variations, were unable to produce significant effects in this case.

The possible changes in brain chemistry resulting from phase shifts, do not appear to have been manipulated in these cases, while benzodiazepines could exert their effects by such a process. This seems a possibility as susceptibility to them occurs at different times of day, presumably reflecting a different mode of biochemical action.

Chapter 8

TIME-OF-DAY EFFECTS ON OPEN-FIELD AND LOCOMOTOR ACTIVITY

8.I Introduction

Tests of "emotionality" or "fearfulness" (e.g. the open field) have been widely used in experimental studies of rodent behaviour. In evaluating open-field data, the usual interpretation is that high emotional reactivity is indicated by a high defaecation rate, low activity or both, while the opposite pattern means the animal is low in emotionality (e.g. Broadhurst, I957; Denenberg, I964; Whimbey & Denenberg, I967). Ambulation in the open field shows little validity as a measure for exploration or crude locomotor activity, owing to the occurrence of both immobility and escape behaviour as alternative forms of emotional behaviour (reviewed by Archer, I973).

Many studies have attempted to find a relationship between emotionality and various other behavioural salients in rodents. For example Furchtgott and Cureton (I964) factor-analysed emotionality and conditioning in mice, while Robustelli (I965) sought the relationship between avoidance-conditioning and maze-learning in rats under noxious stimulation. Similarly Holland and

Gupta (1966) attempted to correlate a conditioned avoidance response with such variables as reactivity and activity.

A previous chapter drew attention to such possible time-dependent variables as stress and activity levels as an important imponderable in the evaluation of passive avoidance results. It therefore seemed of importance to initiate some further experiments to determine any 24-hour variation in reactivity and activity levels as measured by the open field, and by monitoring locomotor activity in the animals' home cage. Furthermore, attempts to correlate individual measures for the open field with passive avoidance scores in the same animal were made and are discussed.

8.2 24-hour variation in open-field behaviour

Following the experimental procedure described in chapter 2, animals were subjected to a one-trial open-field test which involved counting the total line (wall & centre) crossings made in the arena in a 2-minute peridod. Rodents subjected to open-field trials typically orient towards the periphery of the enclosure, and some researchers interprate a high proportion of wall-crossings as evidence of fearfulness. Thus, though these wall:centre proportions were initially differentiated, different groups, it was later found, did not discriminate on the basis of these proportions, and thus no information was revealed. Wall and centre-crossings have therefore been combined to give a total score. Similarly different groups did not differentiate on the basis of urination and defaecation scores, and these results have therefore been omitted.

Care was taken to ensure that all test animals were 7 weeks of age, as age has been found to be an important factor in open-field responsiveness in rodents (Livesay & Egger, 1970; Finger, 1979).

The 24-hour response to open-field exposure is

FIGURE 48 24 HOUR VARIATION IN OPEN FIELD BEHAVIOUR AND THE EFFECT OF CHLORDIAZEPOXIDE

n=I2-I6 ±S.E. ** Differs from non drug treated, p<·02, * p<·05

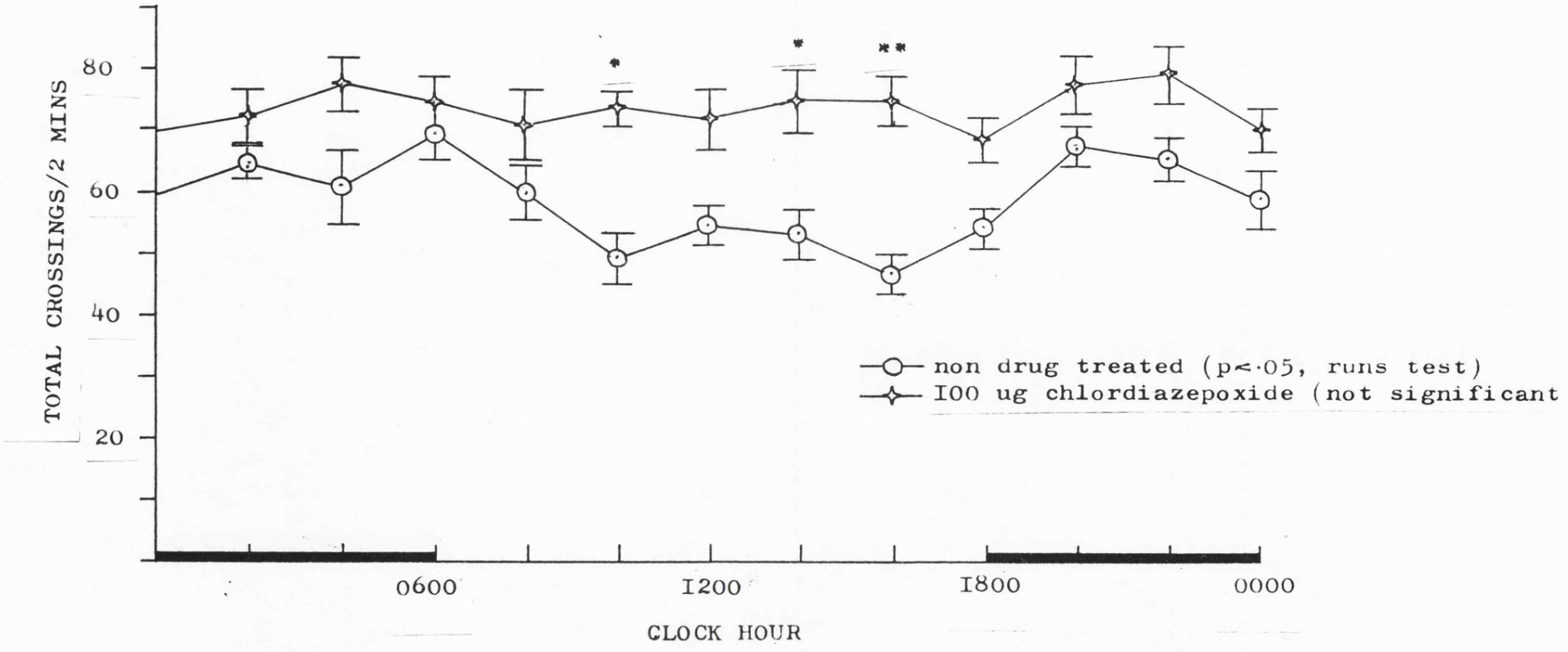


FIGURE 49 EFFECT OF ILLUMINATION ON OPEN FIELD BEHAVIOUR AND THE EFFECT OF CHLORDIAZEPOXIDE

n=12-16 +S.E.

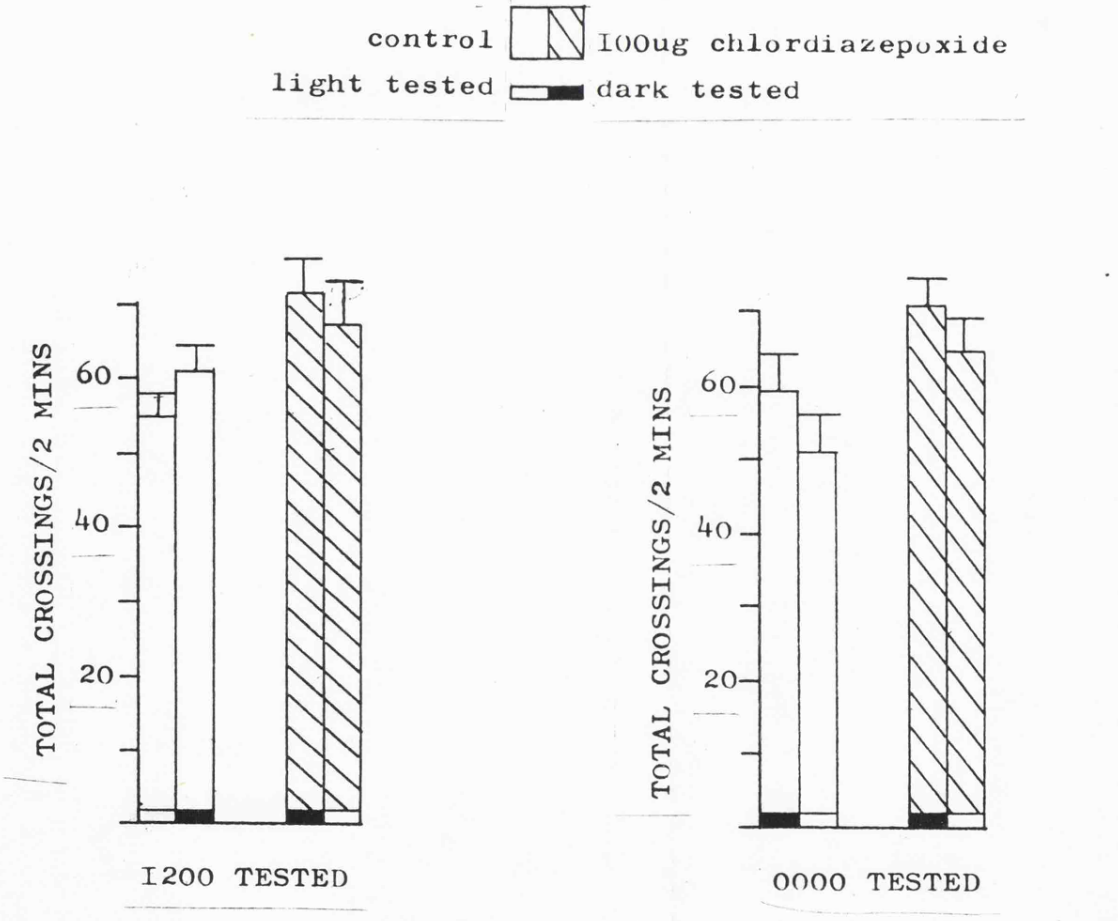
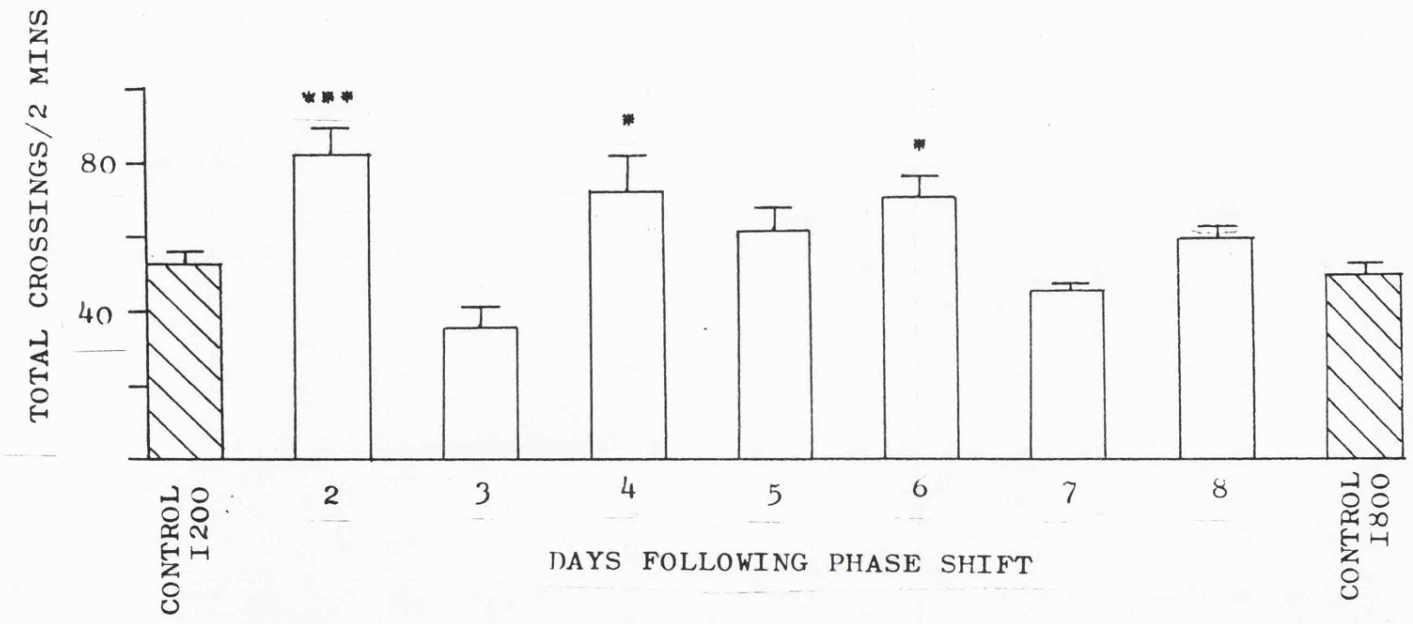


FIGURE 50 EFFECT OF PHASE SHIFT ON OPEN FIELD BEHAVIOUR. CROSSINGS BEFORE PHASE SHIFT AND FOR 8 DAYS THEREAFTER IS COMPARED TO THE 1800 RESPONSE BEFORE PHASE SHIFT. TESTED 1200. n=11-16 ±S.E. *** Differs $p<.002$, * $p<.05$



illustrated in fig. 48, and shows the behaviour to be rhythmic in nature ($p<.05$) and to peak (more crossings) when animals are taken from their light and dark onset, with light-phase animals displaying the lowest tendency to ambulate in the open field. Though the profile bears no resemblance to the 24-hour variation in correct responses for passive avoidance (fig. I2), it must be noted that the sharp decrease in open-field crossing at light onset may parallel the decrease in plate-crossings at light onset (fig, II).

Fig. 48 also shows the open field scores for subjects receiving I00ug/ml chlordiazepoxide administered in the drinking water 24 hours prior to exposure. The results show the drug to increase the total crossings at all times tested, with maximum effect in the light phase, reaching significance (compared to controls) at I000, I400 and I600 hours local time. This is consistent with an anxiolytic drug abolishing fearfulness in animals in a novel environment. Moreover drug administration appears to disrupt the rhythmic nature of the pattern by a "masking effect" where the response in light phase groups is elevated to a comparable level with groups tested at other times. There appears to be little variation and only minor deviation from the overall mean.

8.3 Open-field and passive avoidance correlation

In the next experiment subjects were given identification marks (sample-size I2) and given an open-field trial at II00 hours local time. One hour following this, individuals were given PAR training, and retrial 24 hours subsequently, in an attempt to draw a possible correlation between behaviour in these two situations. If individuals designated high in emotionality (by virtue of low open-field scores) showed a bias towards low passive avoidance scores, then the latter test could be said to depend in part on the

reactivity of the individual concerned.

A Spearman rank correlation test (Siegel, I956) revealed rho=.30I, not significant in the table of critical values. From this it would seem that an emotional state (as measured by the open field) is not a critical factor in determining the outcome of this conditioned avoidance task. This does not of course rule out emotionality factors in passive avoidance, merely that these two experimental methods do not correlate.

8.4 Effects of illumination change on the open field response

Further open-field trials were carried out, involving dark and light-tested groups taken from both light (I200 hours) and dark (0000 hours) environments. It seemed important to confirm that open field trials (which always took place in darkness) were not subject to an illumination change factor, i.e. that animals taken from a light environment were not affected by the sudden change in light intensity. The results (fig. 49) illustrate that illumination-changed groups (i.e. dark-tested at I200 hours) showed slightly more line-crossings than those tested under light (non-changed). Simlarly, at 0000 hours (dark phase), light-tested subjects showed a slight decrease relative to dark-tested counterparts. Although none of these differences reached significance, these small differences are fairly consistent, and it seems that illumination change can cause some small measure of effect in the open field. Whether these small differences result from the actual illumination change, or that ambulation is stimulated by the introduction to darkness is not clear. As previously mentioned, an attempt to circumvent this problem has been made by allowing a I5min. acclimatization period prior to each open field test, in all other experiments.

8.5 Effect of chlordiazepoxide on illumination change

An investigation was conducted to determine whether administration of IOOug/ml chlordiazepoxide in the drinking water, for 24 hours prior to testing, could reduce or increase the small discrepancies in light and dark-tested subjects in the open field, by its known psychotropic effect in alleviating fear. The results (fig. 49) show chlordiazepoxide to raise the total open field crossings in both groups of dark-tested animals (when tested at I200 and 0000 hours) though again not reaching significance. Thus while drug treatment does not appear to discriminate on the basis of illumination (compared to non drug-treated subjects), the overall tendancy of the drug to increase crossings must be noted.

It seems that the drug's anxiolytic properties do not differentially affect animals under illumination change, and that therefore illumination change probably does not constitute a significant stress variable.

8.6 The effect of phase shift on open-field behaviour

A 6-hour advance was implemented at I200 hours local time with daily samplings with different groups of animals from the second to the eighth day after shift. A comparison with passive avoidance was thought to be useful, as any affinities/ dissimilarities with phase shift in the avoidance task, could provide important information on the motivational bases for these behaviours, and whether common links exist between them.

Fig. 50 shows the open-field response in phase shifted animals compared to the expected I800 hours response in controls. A disrupted profile is evident, with maximum effect notable on the second day. Interestingly there exists a trend for increased crossings which increased by a factor of nearly 50% on the second day after

shift. This makes interesting comparison with the PAR where substantial reductions were noted, though this activity followed a similar pattern of disruption and reentrainment. Certainly the converse (i.e. fewer line-crossings) would have been the expected correlate to an increase in emotional reactivity.

These increased crossings in phase-shifted groups could indicate some "unnatural" response to a novel environment, by animals which may increase escape behaviour. Another explanation could be a "disorientation effect" of phase shift, causing disinhibition and possibly actually reducing fear.

As no learning / memory involvement exists in the open-field test, direct comparisons with the PAR are difficult. However if a causal link between phase shift and disinhibition exists, then this could certainly be reflected in increased line-crossings in the open field, and in the inability to respond appropriately to footshock.

8.7 Time-of-day effects on locomotor activity

The daily movements of laboratory rodents maintained in an artificial environment, as measured by such devices as running-wheels, automatic photocells and ultrasonic techniques, have been shown to correlate well with the known nocturnal behaviour of wild species (Barnett, et al, I975). Generally-speaking the mouse is a nocturnal animal, becoming active shortly after dark onset, though the onset of activity may vary by as much as two hours in groups of animals, while a given individual will become active with remarkable punctuality, i.e. animals may synchronize with their peers as well as with endogenous / exogenous time cues (Warden & Sachs, I974; De Coursey, I973).

It is generally clear that activity levels in both

the rat and the mouse tend to wax and wane periodically with dark and light phases respectively (Peacock, et al, I966; Kavanau, I963; De coursey, I96I; I964; Kavanau & Ramos, I975),though differences may result from such factors as age, hunger, thirst and the presence of external stimuli such as noise and temperature.

The introduction of an experimental animal to a test arena always involves such complications as the response to novelty, fear, escape and exploration, all of which will influence activity levels. For this reason, open-field measures are not considered by most investigators as reflective of activity-motivation (reviewed by Halliday, I966; Archer, I973). Conversely automated monitoring of activity levels in the animals' home cage, involving no experimenter-disturbance or additional stressors, can be seen as a more true reflection of the "raw" motor activity levels of experimental groups, while exploration / avoidance parameters can be largely eliminated.

Thus an experimental design following the latter considerations was thought to be pertinent as a comparison with open-field behaviour, and the relative differences in response as a function of time of day. Furthermore, conclusions, it was hoped, as to the involvement or non-involvement of motor activity variation as a variable in passive avoidance could be made. Also any motor response to a phase shift could further enable comparisons of the activity response with that for passive avoidance and open-field behaviour, and to ascertain any similarities in the pattern of reentrainment to the new cycle.

The electromagnetic apparatus and procedure used in the following experiments have been outlined in chapter 2. Briefly, hourly activity counts were obtained from a cage of 25 mice, monitored at 2-hourly intervals throughout 24 hours. This procedure

FIGURE 5I 24 HOUR VARIATION IN LOCOMOTOR ACTIVITY
n=I2 (≃300 animals) ±S.E. significant p<·05 (runs test)

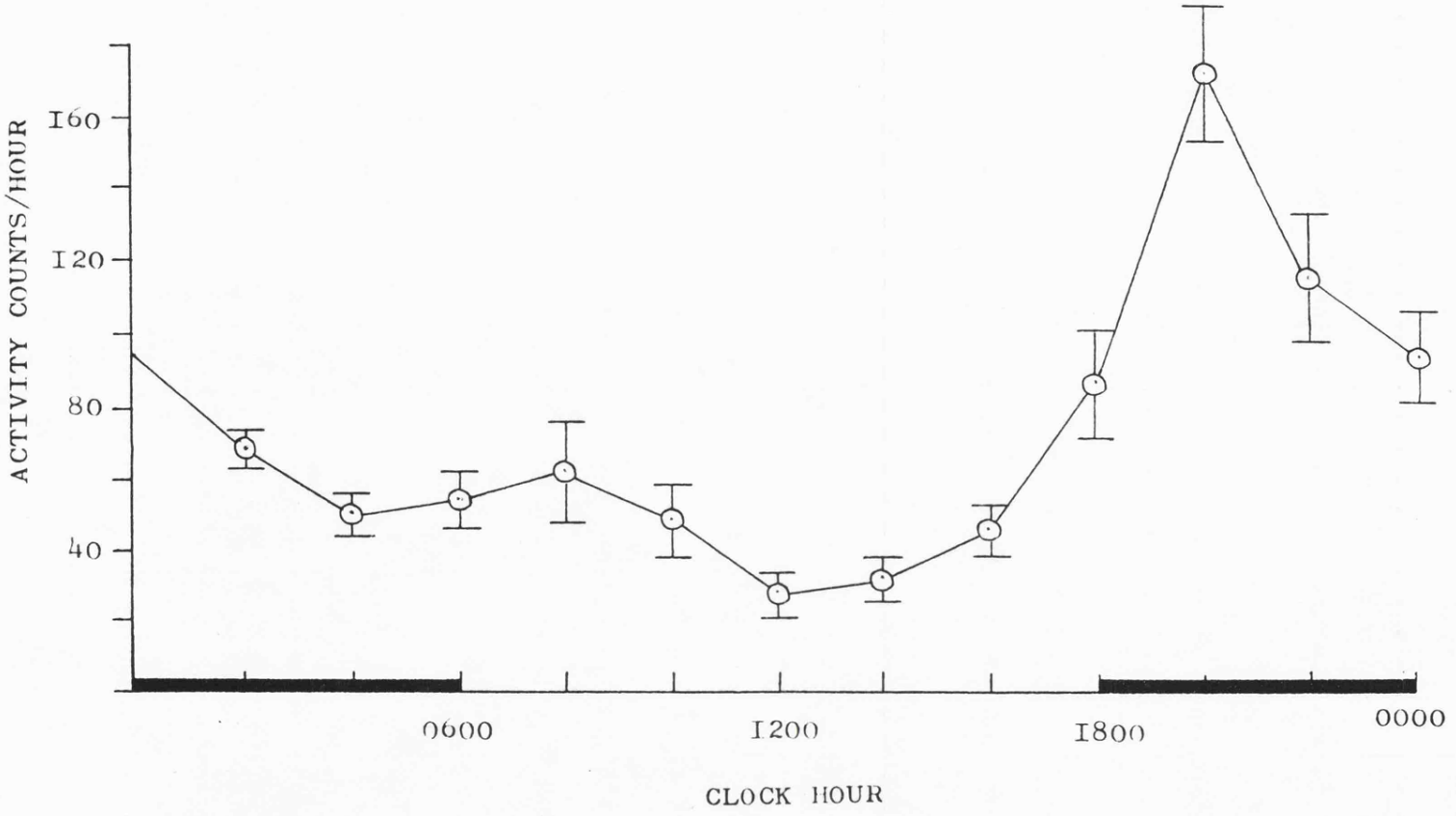


FIGURE 52 EFFECT OF ILLUMINATION CHANGE ON LOCOMOTOR ACTIVITY. n=12 (≤300 animals) ±S.E. *** Differs from light tested group, $p < .002$

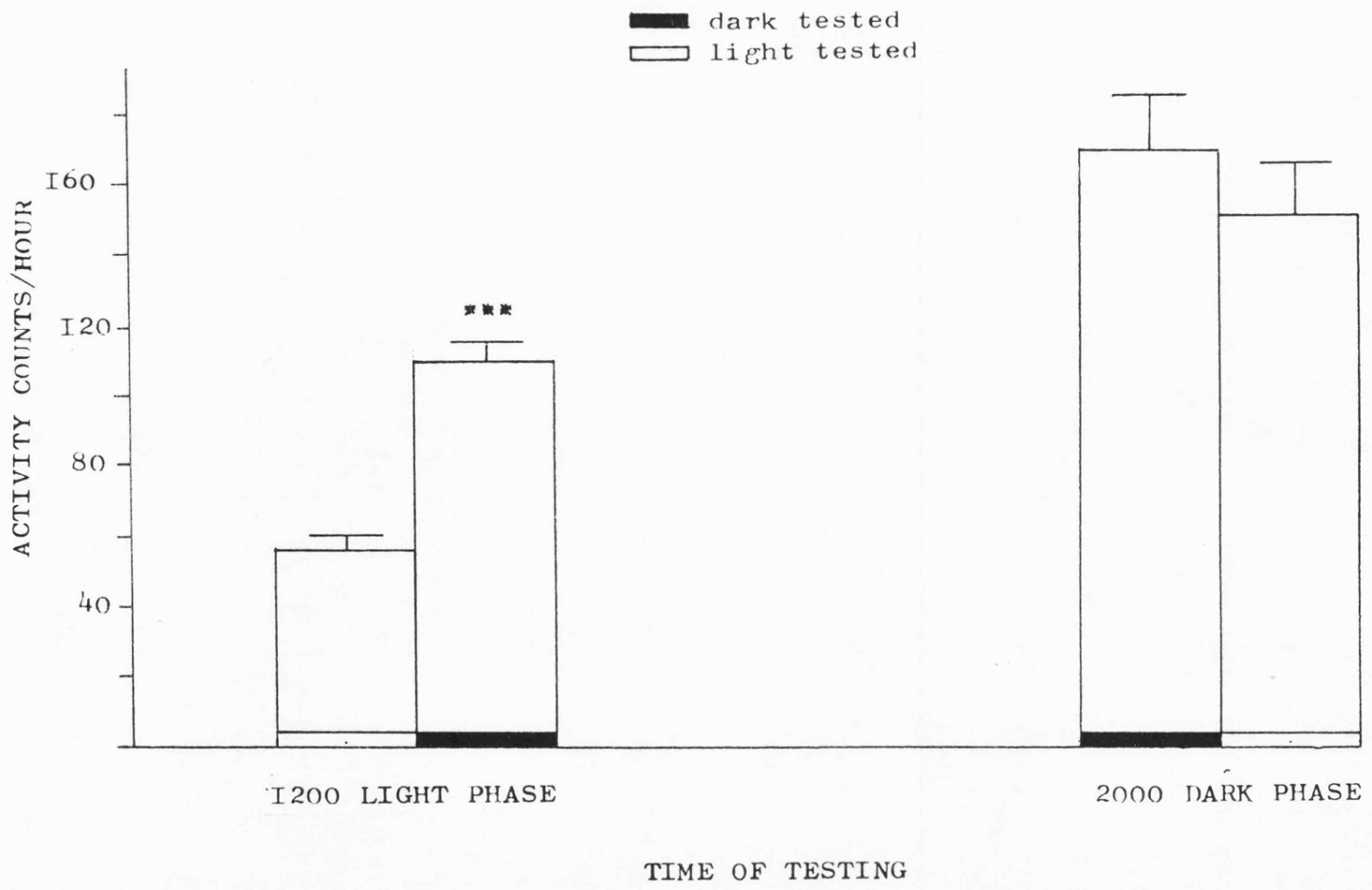
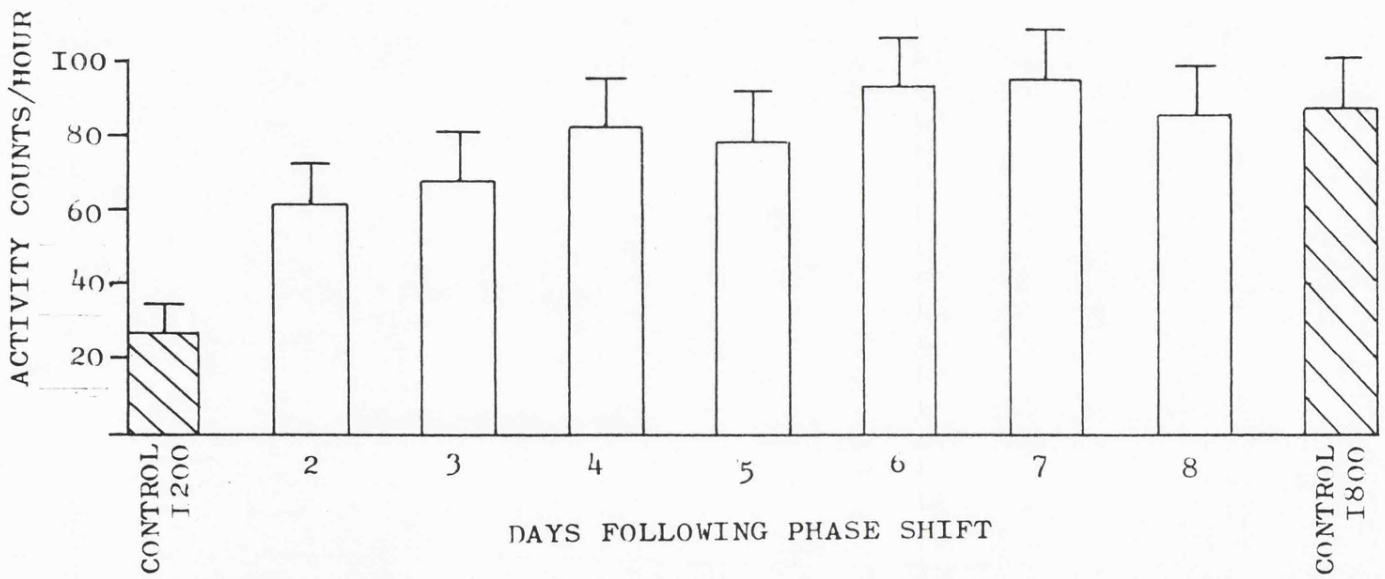


FIGURE 53 EFFECT OF PHASE SHIFT ON LOCOMOTOR ACTIVITY. ACTIVITY COUNTS BEFORE PHASE SHIFT AND FOR 8 DAYS THEREAFTER ARE COMPARED TO THE 1800 RESPONSE BEFORE PHASE SHIFT. TESTED 1200.n=12 (≦300 animals) ±S.E.



was repeated I2 times, involving I2 different cages (25 mice per cage). Thus a total of 300 individuals were employed in the experiment, and these 300 individuals constituted the mean score for each 2-hourly interval.

Experimental subjects were approximately I2 weeks of age, having been previously used in passive avoidance trials. It was ensured that a 5-week interval had elapsed before these animals were used again, though it is conceded that prior experience of an experimental situation is inadvisable in behavioural experiments. However the time-interval before re-use, and their employment in two unrelated experiments, were thought sufficient justification for the re-use of subjects. Also limits to the availability of experimental animals meant that this (and some later) experiment would have been severely delayed, had this procedure not been adopted.

Fig. 5I illustrates the 24-hour variation in motor activity when monitored in the animals' home cage. Activity counts per hour have been plotted against time-of-day, and a significant variation as a function of illumination phase may be noted, peaking strongly two hours after dark onset. Interestingly, both light and dark onset appear to be 'anticipated' to some extent. As expected the pattern of variation bears little apparent resemblance to the open field pattern, though some degree of activity component may be present in the latter, resulting in more line-crossings in dark phase subjects.

8.8 Effect of illumination change on locomotor activity

Though a clear 24-hour rhythm exists for locomotor activity, the extent to which the activity is synchronized with light remains uncertain. As dark onset appears to be anticipated, it seems logical to suppose this behaviour to be controlled endogenously.

Therefore, in order to investigate this, activity levels in the I2 experimental cages were monitored for one hour at I200 and 2000 hours, corresponding to the trough and peak of activity, immediately following an illumination change by the experimenter, from light to dark (made at II:30) and from dark to light (made at I9:30). The results are compared to controls (unchanged illumination), and expressed as histograms in fig. 52. The results show that a sudden extinction of light caused a marked increase in activity, while sudden illumination at 2000 does not result in a significant activity change.

The results are somewhat contradictory in that the animals appear to respond immediately to artificially-induced darkness with increased activity, while failing to respond to sudden illumination with a corresponding activity decrease. The hypothesis may be advanced that while subjects appear to respond immediately to darkness as a cue for mobilisation, their already high arousal level during the dark phase is sufficient to prevent their immediately becoming inactive. It also seems from the I200 result that the animals are responding directly to illumination decrease as an exogenous cue.

8.9 Effect of phase shift on locomotor activity

A 6-hour phase advance was carried out at I200 hours local time with daily monitoring from the second to the eighth day following shift. Results are represented as histograms in fig. 53, and comparisons made with control non-phase shifted groups from the previous experiment. The results show that readjustment to the new expected activity level does not follow the pattern of aberration noted with previous post-phase shift profiles. The resynchronization pattern seems more akin to a smooth 'drift' to the new level, without any apparent transient phase of disruption. The post-phase shift

activity level could be said to resynchronize virtually immediately, as the response on day 2 is not significantly different from the I800 control response.

This result does not conclusively demonstrate that disruption in the 24-hour cycle has not taken place, as more frequent monitoring at different times, over each post-phase shift day would need to be undertaken in order to confirm this.

The results could be interpreted as somewhat at variance with those of post-phase shift monitorings of rats (Navaratnam, I973) which showed resynchronization to activity onset times after 8 days, and also that the amount of activity decreased during the initial 4 days after phase shift.

The virtually immediate resynchronization of activity levels in phase shifted groups may be reflective of their immediate response to light extinction in the previous experiment, i.e. if the animals are largely responding directly to light as an exogenous cue, then one should expect quick reentrainment to the new post phase shift cycle. This makes interesting comparison with the previously-established passive avoidance and open field characteristics, namely a comparative independence of illumination conditions, and a disrupted post-phase shift profile.

8.I0 Discussion

The results reported in this chapter have indicated that 24-hour rhythms exist for both locomotor and open-field activity, though the resultant profiles are not superimposable, and may be thought to be largely unrelated. Individual performance in passive avoidance and the open field has been found to be uncorrelated.

Some measure of illumination-independence has been shown to exist for open field behaviour, while the animals' home-cage

activity was significantly increased by extinction of light. One hypothetical viewpoint is that the direct response to illumination as an exogenous cue may stimulate motor performance, while the relative non-involvement of illumination as a variable in open field performance shows this behaviour to be more reflective of the psychological state of an animal, rather than 'crude' locomotor function. This is further borne out by the finding that a phase shift apparently disrupts performance in the open field, while post-phase shift reentrainment of locomotor activity is relatively quick and free of aberrations, showing 'drift' rather than impairment of re-synchronization.

On the basis of these results, the general hypothetical model may be advanced, of an endogenous oscillator responding only indirectly to light, controlling or influencing those emotional / cognitive anatomical regions governing passive avoidance and open field performance. Conversely, a master oscillator perceiving light as a more direct (exogenous) stimulus, may directly control locomotor activity. This viewpoint concurs with the finding that the more sensitive psychological functions tend to suffer impairment, while the more exclusively motor functions and gross behavioural patterns tend not to suffer from symptoms of desynchronization (Gerritzen, et al, I966; I969). For example the latter author also found drifting of the locomotor activity rhythm.

The difficulties in drawing direct parallels with the human condition have been, and are later discussed, and involve the complication that these models involve only sudden phase shift, while physical translocation and flight stress has not occurred, notwithstanding the social and cultural changes which may additionaly function as a "disorientation variable" in humans (see Aschoff, et al,

I97I). However it seems that the general hypothetical model of an endogenous timing mechanism with an approximate period of 24 hours, but capable of correction by exogenous synchronizers, may be applicable throughout the animal kingdom from unicells to man (reviewed by De Coursey, I973). However further complications arise from the fact that nocturnal mammals tend to undergo long resynchronization periods when readjusting their phase relationship to a new light / dark cycle, while light-active animals generally resynchronize more rapidly, making comparisons between the two difficult (De Coursey & De Coursey, I964; Erkert, I970; Stewart, I962; Rawson, I959).

With these reservations, the open field and passive avoidance response appear to offer themselves as useful animal models in the study of psychological / emotional disturbances resulting from phase-shift induced desynchronization, with the added advantage of total experimenter objectivity, whereas human experimental studies frequently involve 'subjectivity bias' in subjects and frequent misinterpretation by experimenter.

Chapter 9

TIME-OF-DAY EFFECTS ON AGGRESSIVE AND SOCIAL BEHAVIOUR

9.I Introduction

Thus far, the previously-reported experiments have demonstrated the existence of 24-hour variations in passive avoidance, open field and locomotor activities, while motivational relationships between these activities have been sought, together with their degree of dependence upon the exogenous illumination. The expression of these three behavioural parameters are however linked in that they involve no social or asocial component, and by definition are not considered to be social behaviours.

In view of the vast number of physiological factors which vary on a 24-hour basis, it appears logical to predict that social behaviours should also fluctuate with time, particularly if they depend on motor function for their expression. It was therefore decided to investigate some further animal models which, broadly-speaking, examined the degree of time-dependency in the expression of two social behaviours, namely aggressive and (non-hostile) social

behaviour.

The reasons for deciding upon this line of research were threefold, namely (a) to try and demonstrate pattern similarities with open-field and locomotor activity, and to attempt to analyse the relationship with such activities, (b) to establish whether post-phase shift reentrainment patterns for social activities fitted the hypothetical model of an endogenously-controlled oscillation, or whether these behaviours appeared under more direct (exogenous control) influence, responding immediately to zietgeber-change. This could further enable comparisons to be made, and speculations as to whether some aspect of cognitive function is disturbed by phase shift, or whether any observed behavioural changes are more a manifestation of some form of emotional disturbance. Finally (c), if phase shift caused serious interruptions in the stability of measured levels of aggression and sociability, the possibility would exist of drawing parallels with mood-disturbances in phase-shifted humans, with the reservation that extrapolation from this animal model for clinical speculation may be severely criticised.

9.2 Aggressive behaviour in the mouse; experimental considerations

A large number of social species tend to form hierarchies or 'pecking orders'. In these arrangements, dominant animals may have preferred access to food, mates, shelter etc. It is also generally true that aggression in previously-habituated rats and mice shows a gradual decline as the hierarchy is stabilised (Cairns, 1972). Defeated animals tend to adopt the role of subordinates, avoiding, or assuming postures that inhibit further attack. It has been claimed that subordination (Christian, 1958; Chepko-Sade, 1979) has an important role in the dispersal and migration of rodents from over-populated areas. Thus subordination may have evolutionary

importance, as these animals tend to be the pioneers in colonising new areas (Elton, 1942). Also the formation of hierarchies acts to reduce intraspecific damage which would result from prolonged and injurious fighting.

It has been demonstrated that the most common form of social structure in all-male mouse colonies is that of exclusive (or despotic) dominance (Chitty, 1955). In these groups, one or two mice dominate the rest and may attack / threaten all other mice in the colony (Uhrich, 1938; Warne, 1947; Brown, 1953; Crowcroft, 1966). The subordinate mice in turn show little attack on either the dominant mouse or each other, i.e. there is no linear hierarchy or pecking order, c.f. chickens.

In the following experiments, animals were video monitored throughout 24 hours, within the home cage, using infra-red illumination which enabled clear resolution of subjects in total darkness. Thus experimenter-interference was avoided, and the method of investigation maintained as naturalistically as possible. This procedure was thought to yield considerable advantages over other reported experimentally-induced aggression studies (including those concerned with 24-hour rhythms in aggressive behaviour) where unlike these methods involving manipulation by experimenter, the method reported here monitors aggressive behaviour of a spontaneous nature, in the animals' own environment. However it must be stated that the existence of 25 male mice in such close confinement does not constitute a situation pertaining to the behaviour of the animal and would never be found in nature, as the opportunity of escape has been denied.

Experimental animals had 5 weeks previously been employed in passive avoidance experiments, and were re-used for the

reasons previously discussed. The subjects were at least 12 weeks of age at the time of testing. By the use of older animals of this age, one could ensure that sexual maturity had been attained, with consequent aggression-inducing androgen production, together with aggression-releasing urinary pheremones (Dixon & Mackintosh, 1976). Also the elapse of this 5-week interval before re-test, minimised any short-term physiological effects which could have resulted from their use in PAR trials.

The 5-week pre-test period also assured the establishment of a stable colony hierarchy, as attacks are more frequently directed to introduced "stranger" mice (Rowe & Redfern, 1969) and would constitute an additional variable if groups were not comprised of permanently-established individuals. Care was taken to ensure the irregularity of sawdust-bedding replenishment, and that cleaning of cages did not occur for the 8 days prior to testing. This is because the removal of aggression-inhibiting odours in soiled sawdust bedding results in high levels of renewed fighting by subordinates and is a further variable for aggression levels (Dixon & Mackintosh, 1971).

Thus observations of hostile behaviour could be firmly attributed to true social aggression, and not to changed circumstances. It was also ensured that pre-experimental mice were racked in adjacent cages in the holding room and that no experimental rats happenned to be present either adjacently, or in the holding room during this period, as such stressors can influence adreno-cortical function (Bowden, 1979).

9.3 Definition of terms and experimental methods

Classification of postures were based on those originally outlined by Grant (1963) and Grant and Mackintosh (1966).

They are summarised below:

Category	Element
aggression	threat, attack, bite, chase, aggressive groom, offensive sideways, offensive upright
social activity	investigate, nose-nose, attend, approach, groom, sniff, follow, mount, push under, push past, stretch attend.

These 'aggresion'elements were applied by means of a points-scoring system (with minor modifications) originally employed by Valzelli, et al, (I967). The scoring system used to evaluate aggression was as follows:

0= no aggression observed

½= threat (e.g. tail-rattling), assumption of fighting posture, aggressive groom, chase.

I= attack, bite.

By means of the recording time-lapse facility, observations of I5 mins. duration could be made on a 2-hourly basis, and the results from I2 different groups combined (as with locomotor activity). Though the intention was to follow this procedure in monitoring non-hostile social behaviour, considerable difficulties were encountered in applying this method. Unlike aggressive encounters which were overt and obvious, enabling easy discernement on the screen, subtle movements directed towards cagemates were easily masked by the two-dimensional view. An added problem was the huge frequency with which these activities occurred in a group of 25 mice.

It was decided therefore to introduce a system involving paired encounters in a test arena (of the same dimensions as the home cage). Pairs were randomly assigned from cages of stock animals, compared to those employed in aggression tests, i.e. they had previously been used in PAR trials, followed by a 5 week interval. Each test cage was cleaned and replenished with fresh sawdust before each trial, to ensure that no extraneous urinal odours were present, as such odours are by definition "social" and could function as an additional variable. By means of a mechanical timer, behaviour (other than aggressive behaviour) directed to a test partner could be quantified. Thus the total time (in seconds) spent in social activity in a I5min. period, at 2-hour intervals, could be ascertained. Testing took place under both light and dark (under red light) according to the illumination phase of the subjects.

9.4 24-hour variation in aggressive behaviour

Tests for aggressive behaviour using nocturnal rodents are usually carried out during the dark phase of the LD cycle. The rationale is apparently that nocturnal animals will be at their most aggressive at the time when they are normally most active, i.e. at night. It seemed of interest therefore to determine the degree of dependence / independence of this behaviour on locomotion, and whether true rhythmicity for aggressive interactions could be found. The role of illumination as a possible entraining agent was of particular interest.

Experimental animals had 5 weeks previously been employed in PAR experiments and were re-used for behavioural observations. Thus subjects were at least I2 weeks of age when tested, i.e. ensuring sexual maturity and aggression-inducing

FIGURE 54 24 HOUR VARIATION IN AGGRESSIVE BEHAVIOUR
n=I2 (≃300 animals) ±S.E. significant p<·05 (runs test)

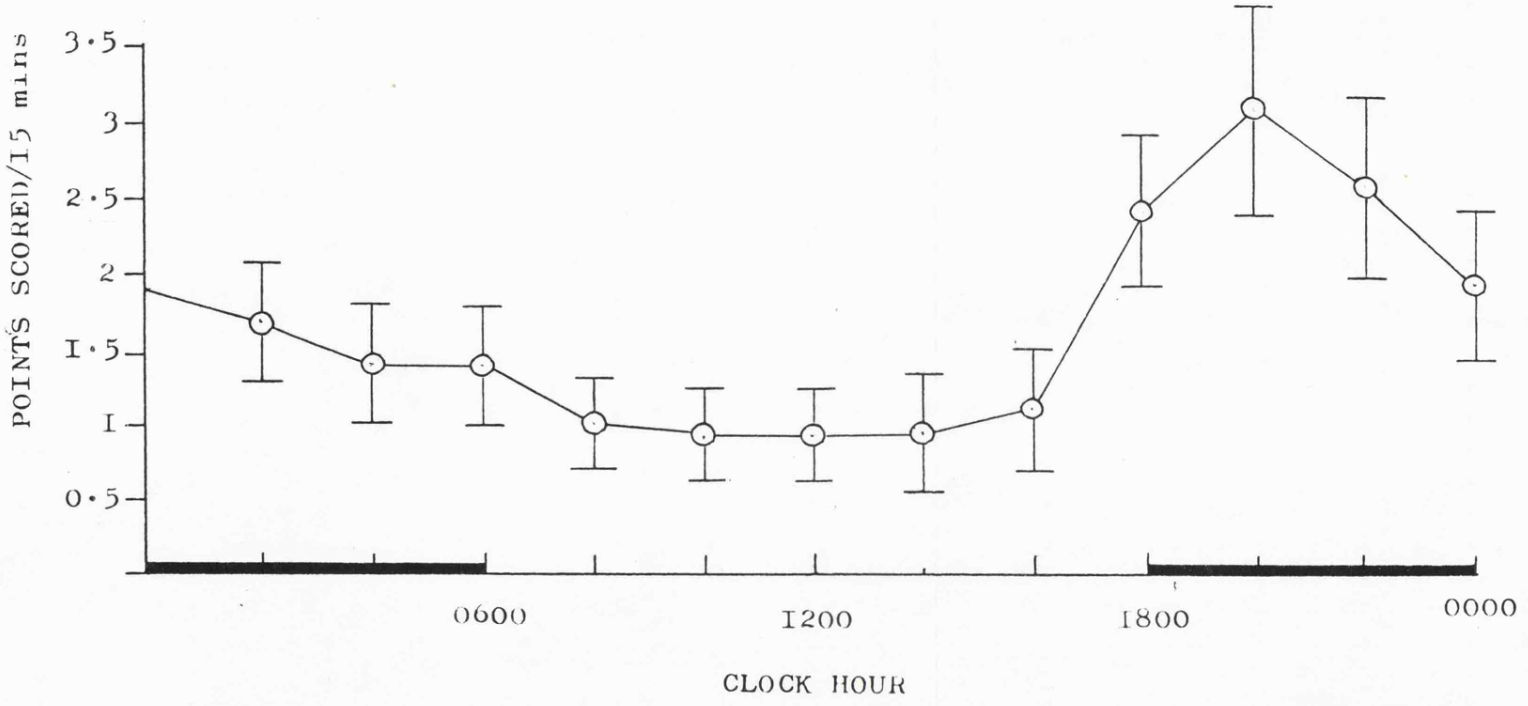


FIGURE 55 EFFECT OF ILLUMINATION CHANGE ON AGGRESSIVE BEHAVIOUR

n=12 (≤300 animals) ±S.E.

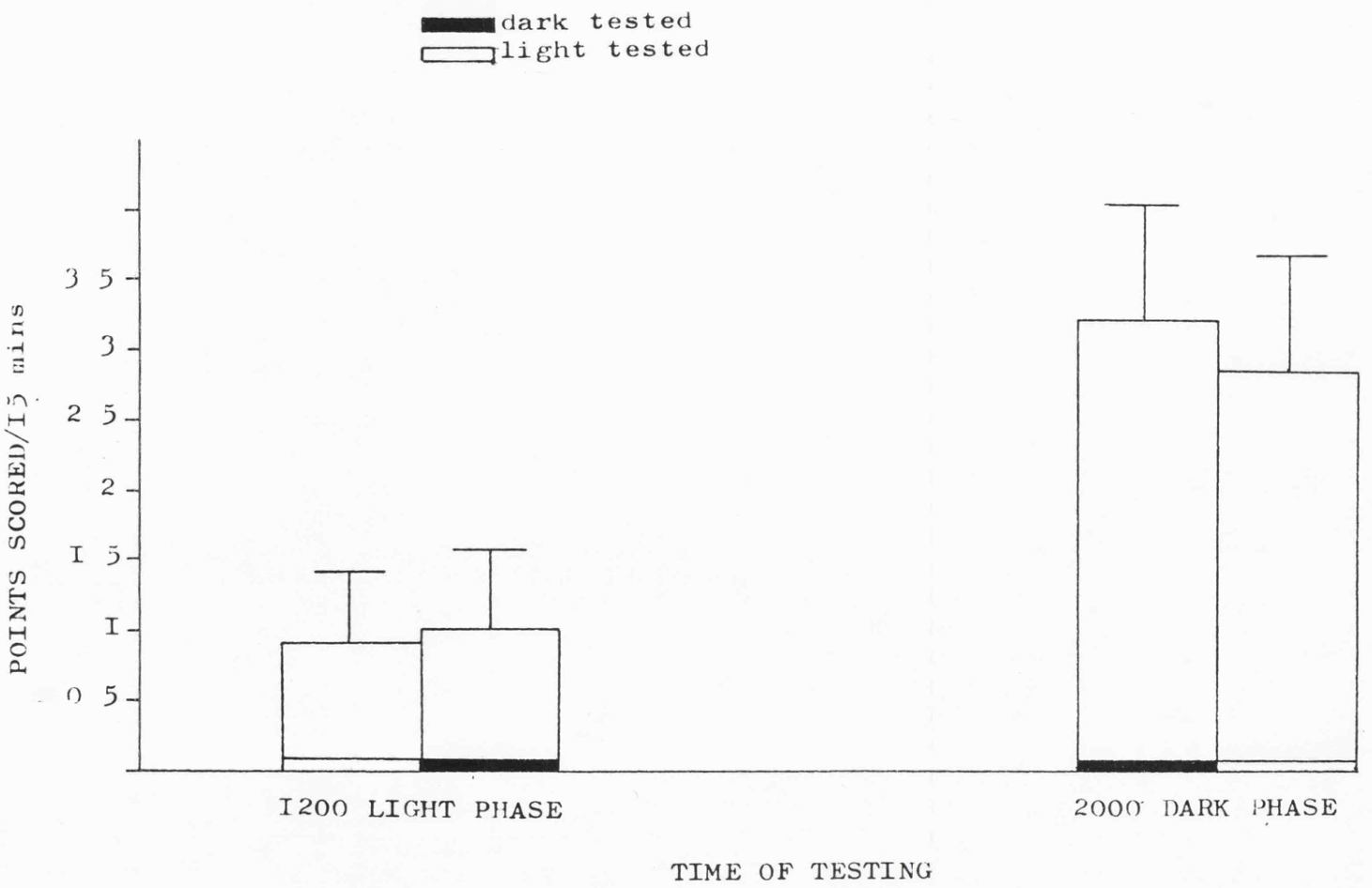
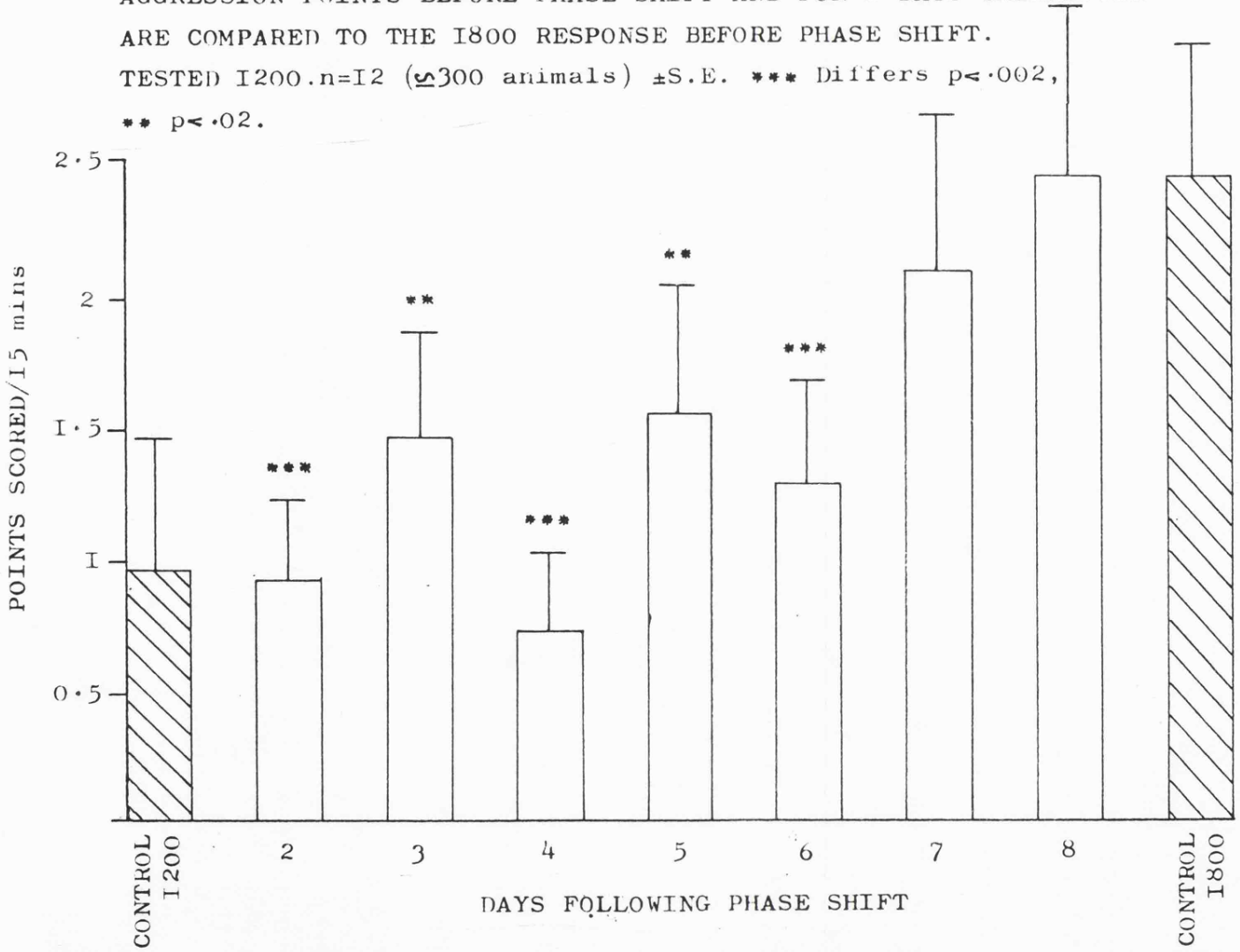


FIGURE 56 EFFECT OF PHASE SHIFT ON AGGRESSIVE BEHAVIOUR.
AGGRESSION POINTS BEFORE PHASE SHIFT AND FOR 8 DAYS THEREAFTER
ARE COMPARED TO THE 1800 RESPONSE BEFORE PHASE SHIFT.
TESTED 1200.n=12 (≦300 animals) ±S.E. *** Differs p<·002,
** p<·02.



androgen production, together with aggression-releasing urinary pheromones / cues (Dixon & Mackintosh, I976). This 5-week interval before re-use also minimised any possible short-term physiological effects of passive avoidance use and further ensured the constitution of stable colony hierarchies.

By means of time-lapse video surveillance in both light and dark periods, aggressive interactions over a I5min. period were quantified at 2-hourly intervals.

The total number of "aggression points" as a function of the time of testing, for each colony (n=I2 results) are illustrated in fig. 54, and show this behaviour to conform to a sinusoidal rhythm, approximately superimposable on that for locomotor activity (fig, 5I) with apparent anticipation of dark onset. This is not surprising as it seems logical to predict that animals should fight more when at their most active, simply by virtue of the fact that increased motor performance is presumably a prerequisite for increased aggressive performance, i.e. the animals' ability to "be" aggressive varies as a function of their motor capability. This result is approximately comparable to those of experimentally-induced diurnal rhythms of aggressive behaviour in mice (Sofia & Salama, I970; Landau, I974a; Ziesenis, et al, I975).

The next step was to try to ascertain the degree of illumination-dependence of this behaviour. Comparisons have been made between groups observed immediately following a light change (from darkness) and a dark change (from light) at 2000 hours and I200 hours, i.e. the peak and trough for this behaviour. Results are expressed as histograms in fig. 55 and illustrate that no between-group difference reaches significance, and that changes in illumination do not elicit changes in fighting occurrence.

The resynchronization profile following a 6-hour phase advance (at I200 hours) follows what appears to be some period of disruption in aggressive responses before resynchronization on the 7th. day (fig. 56). As such therefore, this behaviour does not follow the characteristic reentrainment of locomotor activity (i.e. a relatively smooth "drift"). The hypothesis is therefore advanced that, though dependent upon muscular parameters for its expression, aggressive behaviour is largely pheromonally-mediated and may also fluctuate in accordance with the physiological state of an animal. There remains the possibility of endogenous emotional disturbance, which may parallel the post-phase shift effects in open-field performance.

9.5 Time-of-day effects on social behaviour

For the reasons previously discussed, paired interaction tests were chosen as a method of obtaining measures of possible time-dependency on social activities. A further advantage of this method is that it circumvents the complication of excluding (or otherwise) behaviour associated with the sleep-patterns of the animals. Mice housed in groups typically huddle together when sleeping, and a distinction is normally made between this kind of "passive interaction" and the "active interaction" between dispersed members of a group (File & Pope, I974; De Angelis & File, I979). The dyadic encounter in an unfamiliar arena for a short period of time, is not conducive to sleep / huddling, and more truly reflects active social investigation.

As previously-mentioned, encounters were of I5 mins. duration, at two-hourly intervals, and involved quantifying all activities directed to the test partner, with dark-phase subjects tested in the dark under red light.

The 24-hour variation in social behaviour is illustrated in fig. 57, and shows mathematical conformation to a

FIGURE 57 24 HOUR VARIATION IN SOCIAL BEHAVIOUR.

n=12 (paired encounters) ±S.E. significant p<·05 (runs test)

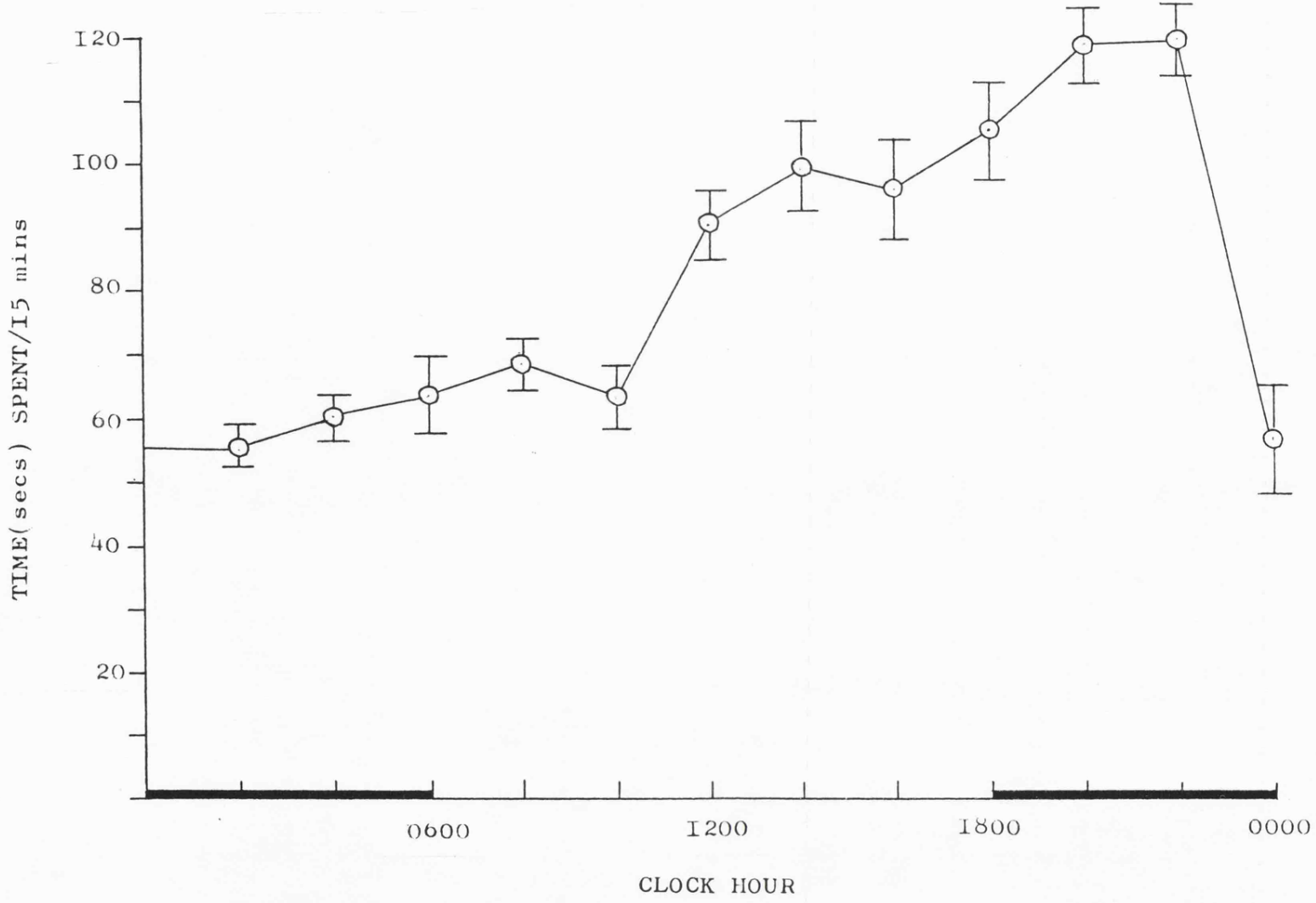


FIGURE 58 EFFECT OF ILLUMINATION CHANGE ON SOCIAL BEHAVIOUR.
n=I2 (paired encounters) ±S.E. *** Differs p<·002, ** p<·02

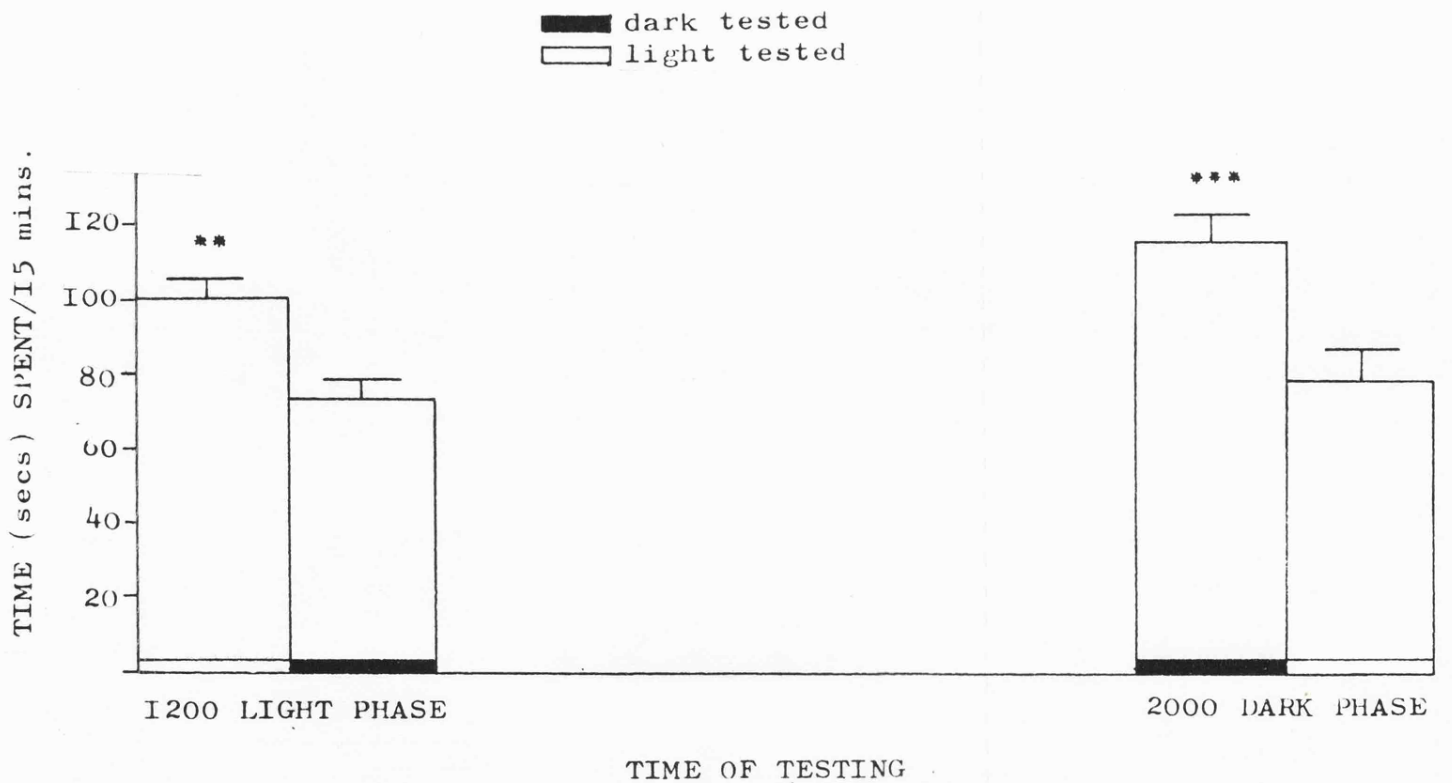
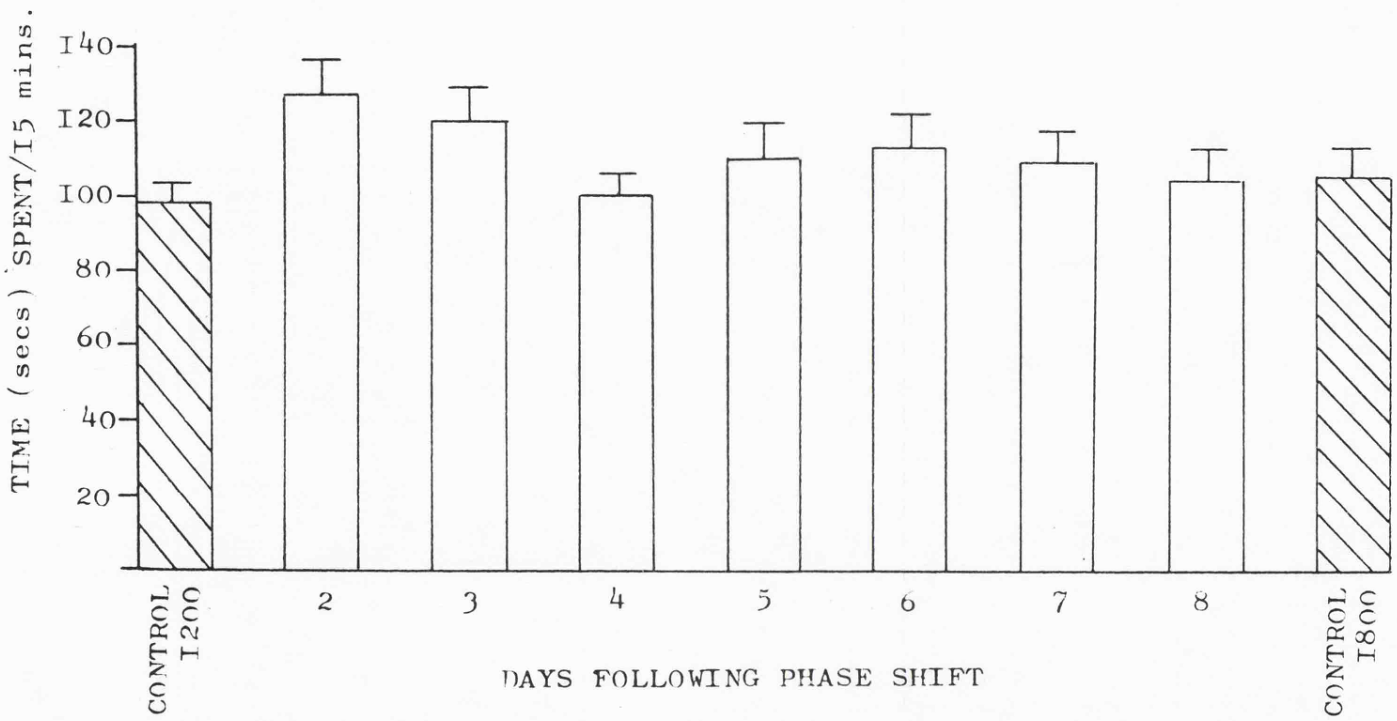


FIGURE 59 EFFECT OF PHASE SHIFT ON SOCIAL BEHAVIOUR.
TIME SPENT BEFORE PHASE SHIFT AND FOR 8 DAYS THEREAFTER
IS COMPARED TO THE 1800 RESPONSE BEFORE PHASE SHIFT.
TESTED 1200. n=12 (paired encounters) ±S.E.



rhythmic oscillation ($p<.05$, runs test), with a gradual increase in response throughout the day. Though the activity peaks strongly following dark onset, it appears independent of illumination, and a sharp increase may be noted at around mid light phase, coinciding with no illumination change. Comparisons with aggressive behaviour (fig.54) locomotor and open field activity reveal no pattern similarities.

Subjects however did respond to the extinction of light with significantly decreased incidence of social investigation (fig. 58). Similarly the sudden institution of light (during dark phase) also resulted in a significant decrease in social activity. At face value these results appear inexplicable in that the animals appear to respond to light as an exogenous cue in their social movements, while on the other hand, the 24-hour variation for this behaviour indicated that no such response to illumination as an exogenous cue was taking place.

It seems a likely explanation that the sudden introduction to darkness may stimulate ambulation and exploration, with a consequent decrease in movement towards a test partner. Also, sudden exposure to light may constitute a stressor which could further direct attention away from the test partner. Thus illumination change may not represent an appropriate model for the investigation of behavioural salients in this case. In brief, a sudden change in the status quo may lead to investigation of the environment rather than of the test partner.

Active social interaction in rats has also been demonstrated to be higher when tested under low light, than in high light (File, et al, I976; File & Hyde, I978), though the latter authors carried out tests in a box with which the animals were familiar.

The post phase-shift resynchronization pattern for social behaviour (fig. 59) reveals no significant deviation from the expected level of response. This corroborates the hypothesis that social behaviour may largely be determined exogenously, though the time-of-day response may involve variations in reactivity to olfactory stimuli or other social cues. The possibility also exists, of differences in anxiety levels, bringing influence to bear upon variations in social activity, where, one could suppose, an increase in anxiety state would decrease investigation of test partners and increase escape-oriented behaviour. Indeed social interaction between paired male rats has been used as a measure of anxiety (File & Hyde, I978).

9.6 Discussion

The results reported in this chapter have demonstrated the existence of a 24-hour rhythm in both spontaneous aggressive, and experimentally-induced social behaviour. Illumination-independence has been demonstrated in the former, while animals apparently respond to illumination change as a social zietgeber.

Phase shift did not measurably induce an aberrant period of reentrainment in social behaviour, whereas some pattern disturbance and time-lag was noted in the readjustment of fighting incidence to the new lighting regimen. Attention has been drawn to the probable relationship between aggressive and motor activity patterns, correlations for which have been found elsewhere (Sofia & Salama, I970; Ziesenis, et al, I975).

The results indicate the aggression rhythm to be largely under endogenous control, though this suggestion is at variance with findings elsewhere. For example,Sofia and Salama (I970) found complete abolition of the aggression rhythm in constant light,

though noradrenaline and 5-HT levels remained the same. These authors suggested the aggression rhythm to be under exogenous control (though they wrongly described the rhythm as "circadian").

Ziesenis, et al (I975), on finding a "biphasic" aggression rhythm in previously-isolated mice, also drew attention to the similarity with the locomotor activity rhythm. These authors also found this "diel" rhythm in aggression to undergo an immediate shift, corresponding to shifts in the photoperiod. Again this would appear to be at variance with the present results, indicating exogenous control.

These experiments do however support the published results of Landau (I974a) who found a true endogenous circadian rhythm for aggressive behaviour in hamsters which was independent of illumination and at least partially independent of the rhythm for non-hostile social behaviour. These results suggest that the aggressive and non-aggressive components of social behaviour both have rhythmic variations while following somewhat separate time-courses.

If as these results suggest, the aggression rhythm is endogenously controlled, this raises the question of possible physiological determinants of the rhythm. Though much research has implicated neural mechanisms (Halasz, I969; Moore & Eichler, I972; Stephan & Zucker, I972) such as the suprachiasmatic nuclei pacemaker mechanism (Moore-Ede, et al, I980; Lydick, et al, I980), in the maintainance of rhythmicity in mammals, the close relation between steroid function and aggressive behaviour suggests a possible secondary endocrine mechanism in the mediation of this behaviour. For example the peak frequency for aggressive behaviour coincides with the approximate peak of measured corticosterone levels in rats (Krieger, I973; Retinie, I973). Adrenalectomy, with or without

glucocorticoid replacement abolishes the nocturnal rhythm in aggressive behaviour (Landau, 1974b), and substantial evidence exists that the expression of the aggression rhythm may depend on circadian rhythmicity of the pituitary-adreocortical axis (Brain, et al, 1971; Harding & Leshner, 1972).

The effect of adrenal and gonadal steroid production on aggressive behaviour has been intensively researched as the most obvious means of modifying social behaviour (e.g. Brain & Bowden, 1971; Heilman, et al, 1976; 1977; Brain & Poole, 1976). However one associated difficulty is the problem of discrimination between hormonal influence on aggressive motivation as distinct from pheromone production, the quality of which mediates aggressive and social responses in mice (Ropartz, 1968; Bronson, 1971; Mugford, 1973; Duvall, et al, 1976). For example, high testosterone production may induce pheromonal changes and consequently lead to more prolonged and vigorous periods of social investigation (Soares, et al, 1978) by another animal, often leading to attack, though the probability is that the more highly androgenized animal will attack first. This illustrates the difficulty in separating motivation from olfactory cues.

This further raises the question of whether the periodic fluctuation in social investigation results from pheromonal changes associated with physiological alterations. Again there remains the difficulty of distinguishing true social motivation from essentially olfactory responses.

Landau (1974a) has speculated as to the adaptive value of the peak in aggression occurring at the time of peak locomotor activity. In social animals such as rats, it may ....."serve to inhibit aggression at times when close proximity would be most

advantageous, i.e. nesting together during daytime rest periods". In non-social species such as hamsters and mice....."it may reflect an increase in overall aggressive readiness at a time when it would be most adaptive (i.e. at dusk and following dark onset), when competition with conspecifics and vulnerability to predators would be greatest". Clearly the importance of the time of testing in determining the exact nature of social encounters may prove of some importance in explaining discrepancies between reports, for which differences in testing time, relative to the illumination cycle were not taken into consideration.

9.4.I The relevance of the animal model

A further explanation for periodic variation in aggressiveness is the variation in the ability of an animal to "be" aggressive. Kleitman (I963) has summarised literature on the periodic differences in simple motor performances in man. In man, these abilities are correlated with the circadian rhythm in body temperature (Schubert, I969). Hence it is possible that observed differences in murine aggression are a function of the normal variation in motor function, i.e. the ability of animals to perform simple motor tasks.

The efficiency of this animal model as a parallel for mood-disturbance in phase-shifted humans may be questioned on the grounds of social / cultural factors and the obviously large phylogenetic gap between the species. However similar correlations between internal desynchronization and emotional stress have been found in both monkeys (Stroebel, I967a) and humans (Winget, et al I97I). Lund (I974), summarising his findings on desynchronizing of of circadian rhythms and personality factors of 34 subjects, pointed out that subjects who became desynchronized had significantly greater incidence of neuroticism and physical complaints. Rockwell,

et al (I974) noted increased depression, hostility and physical symptoms following a photoperiod shift in normal subjects. Altered sleep rhythms are likely to further increase such stress and internal desynchrony.

A partial explanation has been presented by Struve, et al (I972) in that changes in corticosteroid levels appear to interfere with 5-HT turnover (Curzon, I972; Richter, I967). 5-HT neurons are thought to be involved in the control of sleep, aggressive behaviour and pain, as well as playing a major role in depression (Asberg, et al, I976).

The observations presented in this animal study cannot be expected to establish any firm predictive value for internal asynchrony and mood-disturbance in humans. They remain more fundamentally, interesting observations whose clinical significance remains to be fully established

Chapter IO

TIME-OF-DAY EFFECTS OF CHLORDIAZEPOXIDE ON AGGRESSIVE AND SOCIAL BEHAVIOUR

IO.I Introduction

Many studies have attempted to characterise drugs on the basis of their effects on sociability and aggression in laboratory animals. Interest in benzodiazepine effects on aggressive behaviour originated with the early observation that these drugs exert a "taming effect" on otherwise vicious animals (Randall, et al I960). The anti-aggressive properties of benzodiazepines have been confirmed in numerous experimental models, ranging from ants to primates. However many investigators have found decreases in attack behaviour only at doses which induced pronounced muscular relaxation and sedation (Essman, I978; Miczek & Krsiak, I979, for reviews). Conversely several investigators have reported moderate increases in attack frequency in mice, resulting from benzodiazepine treatment (Fox & Snyder, I969; Krsiak, I979; Valzelli, et al, I967), while further variables such as chronic and acute dosage, and use of neutral or resident arenas, have been reported to further complicate findings (Miczeck & O'Donnell, I980).

Overall, the evidence indicates that the

benzodiazepines decrease aggressive behaviour in laboratory animals and in man, while these drugs may heighten attack behaviour at low doses under certain conditions. The reasons for the discrepancies in the literature are manifold, and involve considerations such as different responses by different animal species, and the method used to elicit and evaluate aggression.

Two of the most frequently-used methods for testing the agonistic properties of drugs in mice are the isolation-induced fighting test (Yen, et al, I959; Valzelli, I969), and the shock-induced fighting test (Tedeschi, et al, I959). Both of these methods possess the disadvantage that the aggressive behaviour is induced in conditions which would not usually be encountered in a free environment.

Abnormal brain neurotransmitter function (Essman, I968; Welch & Welch, I97I), has been associated with isolated mice, as has increased adrenal output (Goldsmith, I977). Physiological changes such as these have led some investigators to establish the "isolation syndrome" school (Valzelli, I973), with the assertion that these animals display pathological symptoms of their isolation. The response to drug-treatment may therefore reflect drug effects on these abnormal C.N.S and endocrine responses.

Different neurotransmitters are believed to be involved in the mechanism of aggression (Randrup & Munkvad, I969; Eichelman, I973; Valzelli, I974). Among these many researchers attribute a primary role to dopamine (DA), noradrenaline (NA) and 5-HT pathways in the C.N.S (Gianutsos & Lal, I977; Anand, et al, I977; Kulkarni & Plotnikoff, I978). Conflicting results have been obtained following the administration of drugs which differentially affect brain monoaminergic mechanisms (Hodge & Butcher, I975).

However, most experimental evidence indicates that electrical shock -induced aggression in rats is accompanied by activation of brain DA mechanisms and / or by depression of NA mechanisms (Anand, et al, I977) and that brain NA pathways seem to exert a facilitatory role in male isolation-induced aggression, whereas brain 5-HT pathways seem to exert an inhibitory influence (Hodge & Butcher, I975). Thus it seems probable that a selective pharmacological agent will variably affect different types of aggressive behaviour. This is especially so if one is to apply the"non-unitary" concept of aggression, i.e. that different aggressive behaviours are characterised by different neural mechanisms for their expression (Moyer, I968).

Methods of drug-screening employing stable social groups of animals possess the advantage that aggressive behaviour is not artificially-induced. Thus observed drug-effects on spontaneous aggression may be thought to provide a more reliable measure of the aggression-promoting qualities of a drug, in which one uses a model where physiological changes have presumably not occurred. However, even though a study such as this may provide legitimate characterizations of drugs on the basis of their effects on sociability in laborarory animals, it is still unclear as to the extent to which these pharmacological effects depend on "extra-drug" factors such as social environment and specific inter-relationships, in particular the stability of the dominance-subordinance relationship (Poshivalov, I980).

The aim of the present study was to undertake an investigation on the lines such as has been previously described, i.e. to study the effects of chronic benzodiazepine treatment in a hierarchical organization of animals, with particular interest

FIGURE 60 24 HOUR VARIATION IN AGGRESSIVE BEHAVIOUR AND THE EFFECT OF CHLORDIAZEPOXIDE (broken line)
n=12 (≃300 animals) ±S.E. significant $p<.05$ (runs test)

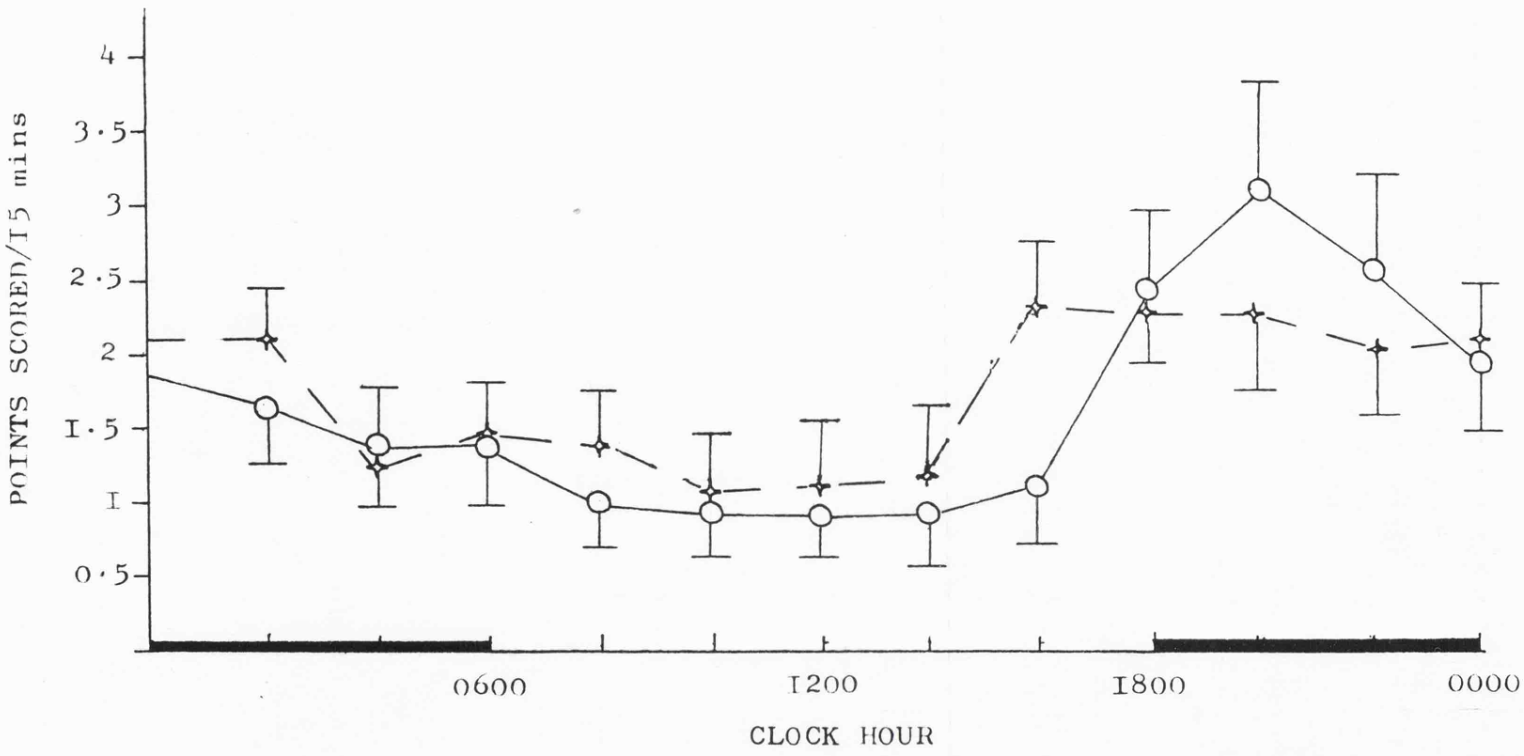


FIGURE 61 THE EFFECT OF PHASE SHIFT ON AGGRESSIVE AND SOCIAL BEHAVIOUR AND THE EFFECT OF CHLORDIAZEPOXIDE (shaded columns). CONTROL NON-PHASE SHIFTED RESPONSE IS ALSO SHOWN (hatched columns) TESTED MIDDAY.

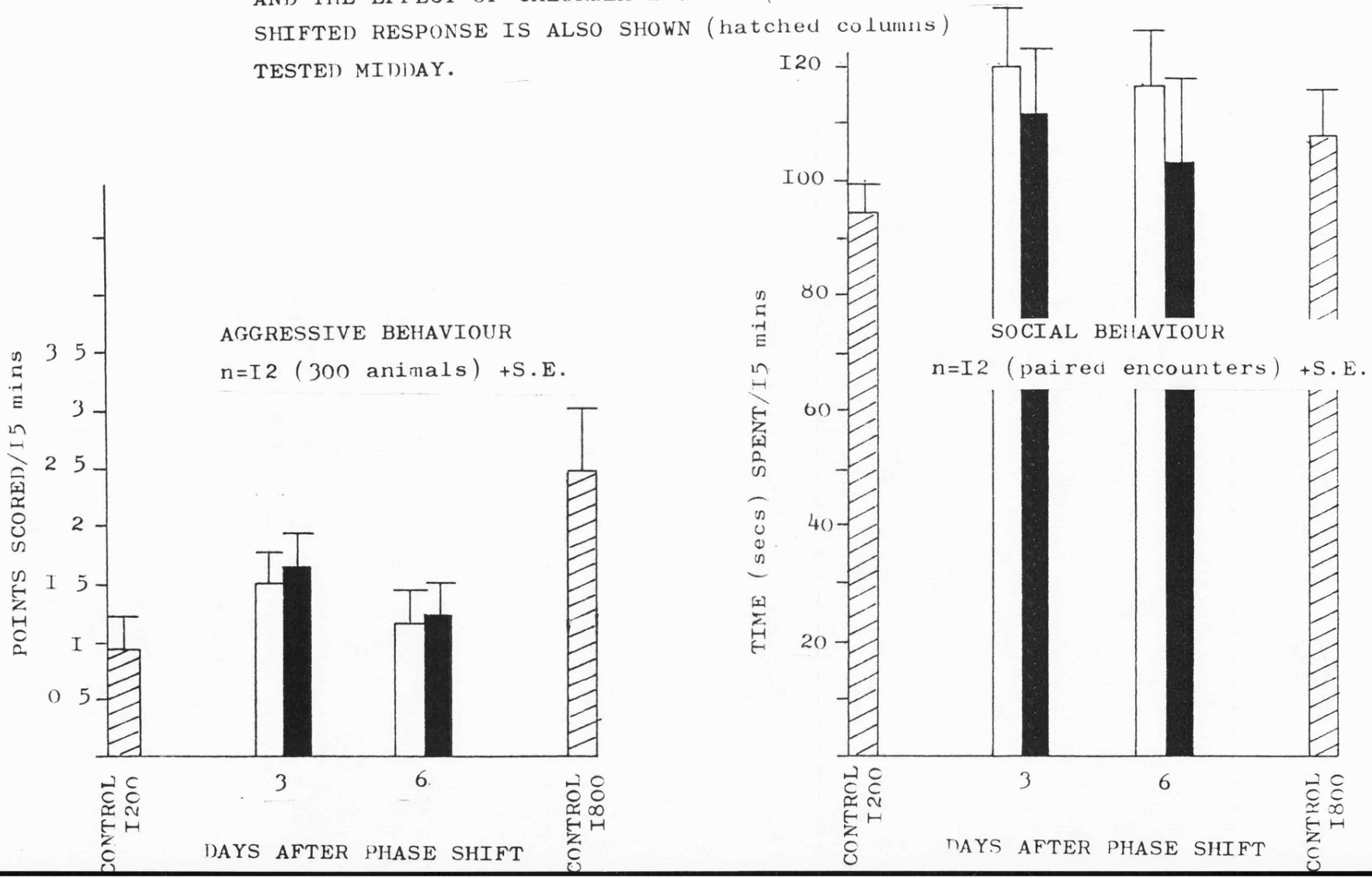
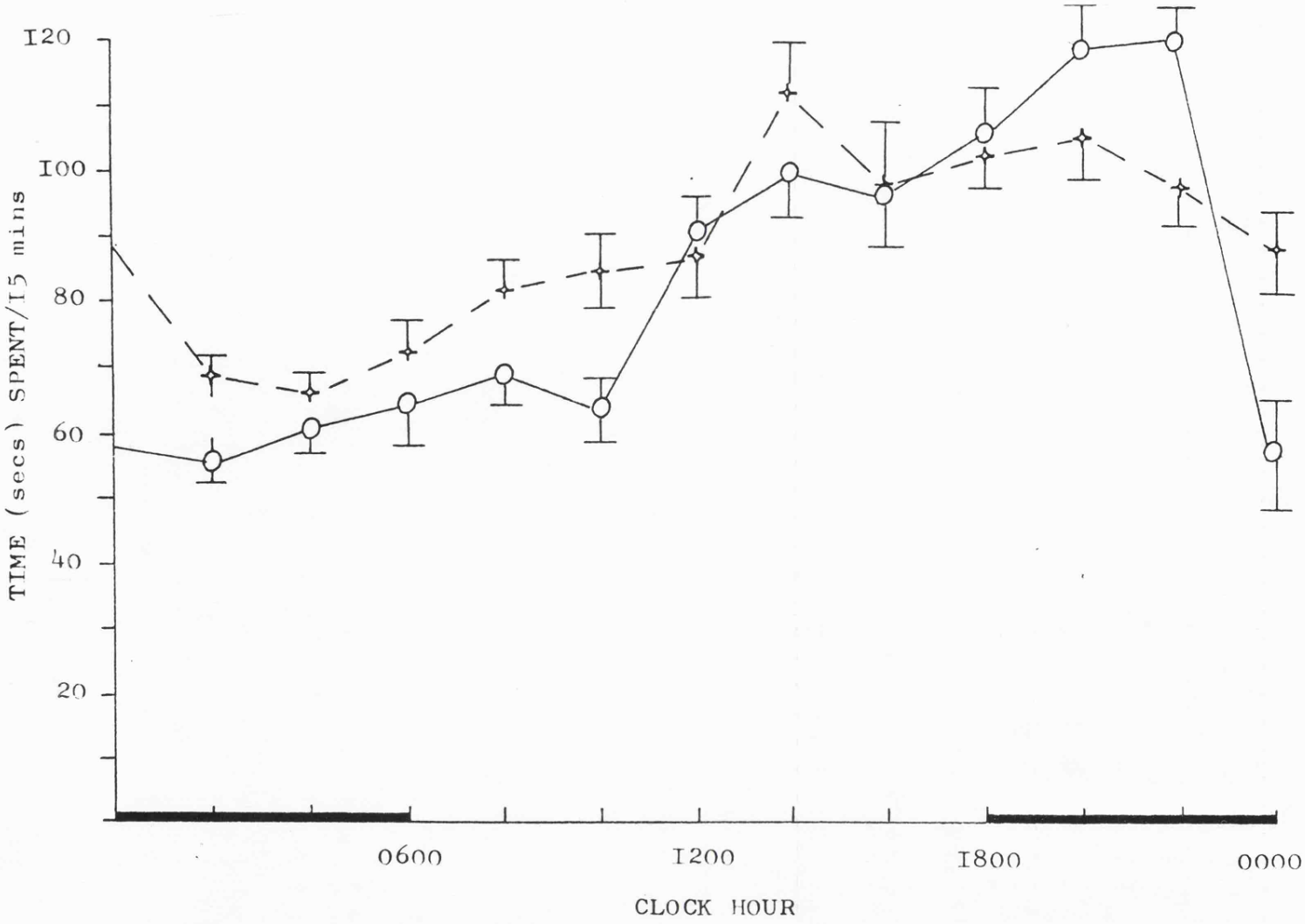


FIGURE 62 24 HOUR VARIATION IN SOCIAL BEHAVIOUR AND THE EFFECT OF CHLORDIAZEPOXIDE (broken line)
n=I2 (paired encounters) ±S.E. significant p<·05 (runs test)



directed to possible variations in drug-response as a function of the time of testing. A further rationale was that if a 24-hour variation in drug-sensitivity to aggression and social behaviour could be established (or otherwise), this would raise the question of the applicability of this design as an anti-anxiety model (c.f. File & Hyde, I978), enabling comparisons with patterns obtained from the open-field and passive-avoidance data.

IO.2 Time of day effects on aggression

The experimental design, method of surveillance, and points-scoring system were exactly as outlined in the previous chapter. In this case, however, IOOug/ml CDZ was administered to animals in the drinking water, for at least 24 hours prior to observations.

The effect of IOOug/ml chlordiazepoxide treatment on aggressive behaviour is illustrated in fig. 60 and shows the drug to reduce the incidence of aggression during the dark phase (peak level), while no substantial effects may be noted at other times. It seems a feasible argument that this may not reflect a pronounced variation in sensitivity to the drug, so much as a more easily reproducible effect of the drug at peak aggression times, compared to times when little fighting occurred. As disproportionately few (2 or 3) individuals in each group did most of the fighting, this could possibly present a false effect as certain individuals may respond more to the drug than others.

Though reducing the rhythmic nature of this behaviour conformation to a rhythm is still maintained, and as such shows dissimilarity with open-field data where chlordiazepoxide was shown to abolish the 24-hour oscillation of that behaviour. It is therefore possible that motivation for open-field movements and

aggressive behaviour are unrelated, i.e. the aggression model may not constitute an appropriate test for anxiety.

Aggressive responses on the 3rd and 6th after phase-shift, monitored at midday, with groups maintained on IOOug/ml chlordiazepoxide treatment (fig. 6I), are not significantly different from controls and indicate that the drug does not facilitate resynchronization, unlike its effect on passive avoidance.

IO.3 Time-of-day effects on social behaviour

Apart from the open field, two tests have traditionally provided animal models of anxiety; the conditioned emotional response (Estes & Skinner, I94I) in which anxiety is equated with conditioned fear, and the rat conflict test (Geller & Sefter, I960). Both tests involve deprivation and electroshock, and any experiment involving drugs must incorporate controls for drug-induced changes in motivation or in sensory thresholds.

A useful alternative test for anxiety, involving neither deprivation, nor electoshock has recently been developed (File & Hyde, I978), in which social interaction between paired rats placed in unfamiliar surroundings, was used as a measure of anxiety, and was thought to constitute a legitimate "screen" for anxiolytic drugs. It was thought to be of interest to apply this model as an anxiety-reduction test for chlordiazepoxide, using data obtained from the paired interaction tests in the previous chapter as base-line comparisons.

In this experiment, IOOug/ml CDZ was administered to the animals for at least 24 hours prior to observations, via the drinking water, contained in light-proof bottles in the animals' home cage. Experimental methods were exactly those described in the

previous chapter, involving paired interaction tests in a neutral arena, following removal from the home cage.

The results illustrated in fig. 62, show that the overall effect of the drug is to slightly raise the time spent in social investigation, throughout the day, though slightly decreasing relative to non-drug treated controls at dark onset. At face value, it seems that the animals are less stimulated to socially investigate when drug-treated and under darkness. However it seems feasible to suppose that drug treatment raises the anxiety threshold when animals are tested under light (bright light probably constitutes a stressor), while drug-treatment fails to raise anxiety thresholds to a comparable degree when subjects are tested under darkness, to to their already high "social motivation".

The response to phase shift in chlordiazepoxide-treated mice (I00ug/ml; fig. 6I) is not significantly different from that of controls, and it therefore seems that if this phase shift model does provide for an index of an anxiety state, the anxiolytic properties of the drug are not acting in this case.

IO.4 Discussion

The results reported in this chapter demonstrate that chlordiazepoxide has no clearly-defined action (reducing or increasing) on aggressive behaviour when sampling is taken throughout 24 hours, though a reduction in response at the peak aggression period (after dark onset) takes place.

It is unclear whether this represents heightened sensitivity to the drug at this time, or whether some other behavioural factor is responsible for the reduction, e.g. C.N.S action may block the expression of aggressive behaviour by disrupting goal-directed co-ordination of movement (see Crowley,

I972), or peripheral factors such as responsiveness to aggression cues may be differentially affected over time of day.

Drug-treatment apparently caused heightened social investigation (reduced anxiety?) in the early part of the day. Interestingly this bears comparison with the variation in drug sensitivity in the passive avoidance test, which also demonstrated increased susceptibility to drug treatment, generally at the early part of the day.

As with aggressive behaviour, drug treatment induced a decline in social activity in the early part of the dark phase, relative to groups receiving no drug. Because of this characteristic similarity of the two models, it seems logical to suppose that the two models may have parallels with each other, in that some broad aspect of social behaviour may be indirectly or directly influenced, rather than C.N.S sensitivity to drug-treatment.

As drug treatment evoked no behavioural response (aggressive or social) in phase-shifted mice, the phase-shift models in this context are not of particular interest. It is however interesting to relate that because social responsiveness (undisturbed by phase shift) and aggression (disturbed by phase shift) did not respond to drug-treatment, this would tend to indicate that similarities exist between the two models, and further underlines the importance of "social considerations" when making judgements as to emotional / anxiety states.

Aggressive and social behaviour, it is concluded, remain questionable as to their utility as an animal (murine) model with clinical application, due to the vast array of social imponderables presented. These studies, it may be argued however,

are worthwhile as comparisons with other models used in this thesis in terms of their particular characteristic adaptations to changed environmental circumstances, when treated with centrally-acting drugs.

Chapter II

GENERAL DISCUSSION

II.I Introduction

The previous discussions have dealt with specific areas of study, and relate to each individual chapter, though some speculative comparisons are made. The aim of this discussion is an attempt to relate the different areas of investigation and to establish a "macroscopic" viewpoint.

The rationale for this thesis has been an attempt to draw parallels between the known psychological disturbances in human beings following physical trans location, with changes in the amplitude of various animal responses as a function of the time of testing, and of changes in environmental zietgebers. The possibility of pharmacological manipulation or prevention of these symptoms forms an important dimension to these experiments.

It has therefore been necessary to establish the existence of 24-hour variations in simple (putative) learning and retention tasks, together with aspects of time-dependency on the expression of some further relevant behavioural parameters. Again, the time-of-day susceptibility to a number of clinically-important drugs has been of paramount interest. Because this thesis has centred on a large number of

drug manipulations and behavioural comparisons, it has tended towards a broadly-based study, rather than an in-depth examination of a narrow field.

Finally, in the light of these findings, suggestions are made as to the possibility of further valuable information being obtained from future experiments.

It has become increasingly clear that the different behavioural parameters studied, display different characteristics, and respond in different ways to manipulations of the environment. The conclusion has therefore been that the various areas of behavioural study may vary according to different biological systems. These biological systems may be conveniently divided into those which appear to be directly attuned to alterations in the environment, and those which oscillate in sympathy with external factors , which require some time to re-adjust when these external factors are changed. The latter seems to represent the most commonly-described rhythm in the literature.

II.2 The entrained endogenous model

Following a sudden phase-shift of the environmental synchronizers, the phase relations between these synchronizers and the internal rhythm also shifts. Rephasing of these components may be attained only gradually, and several days may elapse before complete re-entrainment of the self-sustaining rhythm and attainment of stability. In contrast, a passively-driven rhythm would be expected to show virtually immediate resynchronization.

Thus, the 24-hour variations in passive avoidance, open field, and aggression display the characteristics of an internal rhythm under external synchronization because:

(a) They oscillate with sinusoidal regularity.

TABLE I

THE EFFECTS OF ENVIRONMENTAL MANIPULATION ON 24 HOUR BEHAVIOURAL RHYTHMS

			post-phase-shift characteristics	
behaviour	non-random sequence	illumination independence	response amplitude	resynchronization profile
passive avoidance	yes	partial	reduction	disruption
open field	yes	yes	disruption	disruption
aggression	yes	yes	disruption	disruption
sociability	yes	no	unchanged	drift
locomotor activity	yes	partial	unchanged	drift

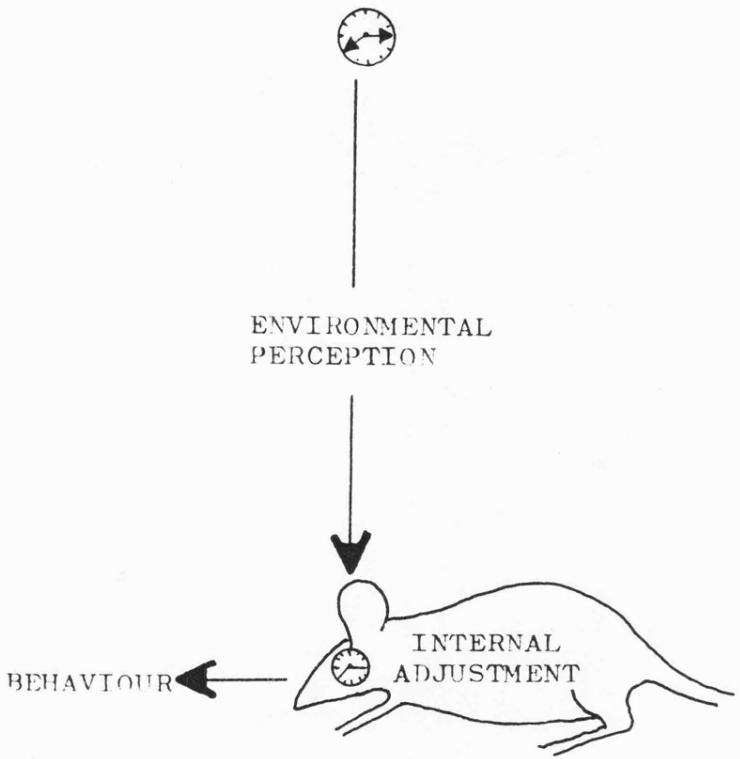
TABLE 2

THE RELATIONSHIP BETWEEN ALTERATION OF PHASE AND RESYNCHRONIZATION INTERVAL FOLLOWING A 6 HOUR PHASE SHIFT

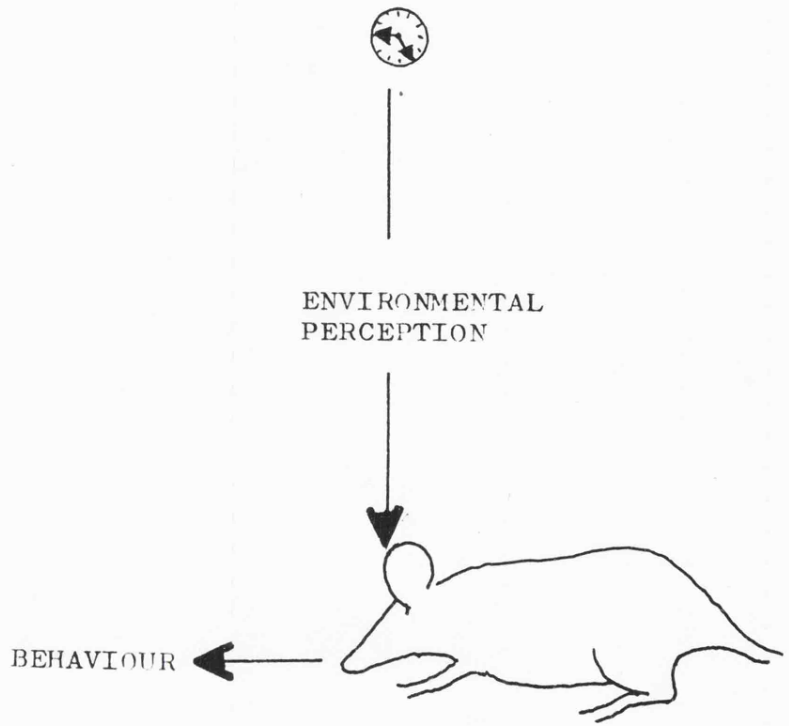
behaviour	change of phase (%)	resynchronization interval
passive avoidance	77·2	6 days
aggression	69·5	7 days
locomotor activity	44·4	none
sociability	22·9	none
open field	4·6	7 days

FIGURE 63

THEORETICAL MODEL FOR THE ENDOGENOUS RHYTHM UNDER EXTERNAL ENTRAINMENT



THEORETICAL MODEL FOR THE EXOGENOUS RHYTHM



(b)They display either partial or complete independence of the environment (illumination).

(c) Following a change in the environmental zietgeber (illumination), the resynchronization profile shows reduced amplitude and reduction of the overall mean (i.e. the oscillation may be "damped")or disrupted, or both (see table I & fig. 63).

These behaviours may thus be said to conform to the acceptable definition of the term "circadian rhythm", though further testing under constant environmental conditions would be needed to establish these responses as "free running", and totally endogenous to the organism.

II.3 The exogenous model

The 24-hour variations in social and motor activity conform to (largely) exogenously-determined rhythmic processes because:

(a) They fluctuate with regularity in accordance with periodic changes in the environment.

(b) They display either partial or complete illumination -dependence.

(c) Following a change in the controlling environmental zietgeber, these responses immediately re-adjust to the altered circumstances, and do not show reduced amplitude or disruption (see table I & fig. 63).

II.4 Anticipation of photoperiod change

Another characteristic of endogenously-controlled biological rhythms is their ability to "anticipate" zietgeber change. An example is the rhythm for human body temperature which starts to increase from its low night-time level before the end of the dark phase and before the subject has awakened (Aschoff, I970).

In the previous experiments, aggression and locomotor activity showed appreciable increases which preceded dark onset, though

the latter behaviour is still referred to as "exogenously-controlled". This apparent inconsistency however, seems less significant on considering reported literature which show animals to demonstrate temporally-conditioned changes and physiology preceding a periodic stimulus, which cannot be attributed to an endogenous oscillation (Boulos & Terman, I980). For example anticipatory changes have been associated with fixed interval schedules of reinforcement (Ferster & Skinner, I957). Thus while the occurrence of anticipatory changes may be suggestive of endogenous entrainment, they do not necessarily presuppose such a process. Boulos, et al (I980) have reported that rats may show anticipatory lever-pressing when subjected to 24-hour feeding schedules. The authors interpreted these findings as indicating that while the timing of anticipatory behaviour itself may be mediated by its own distinct circadian mechanism, this process is distinct from other circadian mechanisms of behavioural entrainment.

It is interesting that chlordiazepoxide has been found to enhance food-maintained responding (Bacotti & Barrett, I976), indicating that the drug could possess some direct circadian effect in this case.

II.5 Phase shift

Phase-shift-induced disturbance may vary according to:

(a) The amount (or degree) of shift.

(b) The direction (advance or delay) of shift.

However phase delays and shifts of other than 15^{o} have not been the concern of this thesis, and they have not been attempted. There are however other variables associated with the 15^{o} advance (employed in this thesis) and they are chiefly concerned with the phase of the oscillation at which shift occurred.

In an attempt to maitain inter-experimental consistency

all phase shifts were instituted at I200 hours local time, which coincided with the mid light phase of the animals LD cycle (all shifts were advances of 6 hours). However acrophase at one time of day for one behaviour will not necessarily correspond with another, as different 24-hour oscillatory profiles appear to exist for different behaviours. This would of course mean that the institution of phase shift may occur at any phase, involving a possible peak, trough, upward or downward gradient for the behaviour under study. This would appear to constitute a variable as to the degree of phase shift in each case. Aschoff (I960) has stated that each sudden upward or downward step of the level of oscillation results in a phase shift, and further, that the amount of shift depends on the phase at which the level had been changed, and on the difference between the two levels.

At face value this statement means that any hypothetical phase shift of 6 hours which happened to coincide with successive reccurrences of of a particular phase of a cycle would involve no phase shift! However Aschoff has further stated that each change of speed for a given fraction of period does constitute a phase shift. Thus the longer the system runs faster or slower, i.e. the longer the light time or dark time, the greater the advance or delay respectively.

II.6 Determination of amplitude change

In consideration of the possible complications mentioned in the previous paragraph, it seemed of great importance to try and provide some kind of index of the amount of change occurring when I200 became I800 as a result of phase shift. An appropriate method was thought to involve calculation of the difference between the two levels, expressed as a percentage of the total rhythm

amplitude (i.e. the difference between peak and trough). Thus indexes of amplitude change are calculated by the formula:

$$\frac{\text{Difference between I200 \& I800 level}}{\text{peak - trough level}} \times 100$$

The results are shown in table 2, in relation to the respective resynchronization period (defined as the time taken to achieve levels not significantly different from the I800 control response). It can be seen that although phase shift in parameters such as the open field involve only 4.6% change, a considerable time-lag is apparently required before re-entrainment is achieved, and it therefore generally appears from these results that a phase shift as defined by these experiments does cause disruption and lengthy resynchronization requirements, and it appears not to be the case that a substantial change of acrophase is necessary to achieve these effects.

II.7 Phase-shift in humans & relevance of the animal model

It is generally-supposed that rapid changes in environmental zietgebers cause an "upset" to entrained biological systems and may result in some form of stress to an organism. The present findings appear to show (and confirm) that sensitive psychological parameters (if one includes aggressive behaviour) may manifest this "stress", while the less sophisticated motor patterns appear to be relatively unaffected.

II.7.I Arousal and emotional factors

It is now well-documented that short-term memory is impaired under conditions of high generalised drive or arousal level (Kleinsmith & Kaplan, I963; Walker & Tarte, I963; McLean, I969). Using human subjects, it has been demonstrated that performance on tasks involving high memory load, with little immediate processing vary as an inverse function of the arousal level of the brain, according to the time of day (Colquhoun, I97I), i.e. as arousal increases

throughout the day, so performance on the immediate processing components of a task improves, while performance on the memory component declines.

If the latter were applicable in the murine passive avoidance context, this could explain the paradoxical effect of achieving the lowest result when the animals are at their activity peaks. It is however, extremely difficult to isolate variables of possible emotional origin. Emotional states will vary with daily physiological changes within the organism, and in turn influence motivation and motor responses. Furthermore any state of excitement or peturbation will inevitably disturb the functioning of intellectual processes. There is the additional complication that emotional / fearful animals tend to show either a freeze or flight response when introduced to a novel test situation, or noxious stimuli. Both the latter responses would undoubtedly detract from accurate assessments in passive avoidance trials. In strange situations the performance of the emotional animal is inferior to that of the less emotional animal, because fear inhibits the ability to learn and to make adequate adjustments to changed circumstances.

Factors such as these are virtually impossible to account for in an experimental situation, such as that of the avoidance-learning task. Clearly therefore other measures were required in order to provide a more accurate assessment of the time-of-day effects on an animal's emotional state, and it was hoped that an examination of open-field and social responses would provide this assessment.

It is fairly certain that physiological conditions of arousal are connected with increased exploration (Halliday, I966). Thus, high ambulation in the open field presumably varies as a

function of arousal, whilst being negatively correlated with fear. Applying this viewpoint, it thus appears (from the open field data) that arousal may be at its lowest point during the light phase, while showing an increase (and fear decrease) at dark onset, while a decrease may occur at the approach of light onset.

The opposite may be noted with passive avoidance retention. This would appear to be consistent with the idea that memory processes vary inversely with, and are inhibited by drive.

With respect to passive avoidance, it has been suggested in a previous chapter that pain thresholds may also show time dependency. Both circadian and seasonal variations have been detected for stimulation-induced analgesia in rats (Buckett, I98I), while diurnal variations in tolerance to painful stimuli have been correlated with brain enkephalin levels (Wesche & Frederikson, I980). Experiments do not account for the possibility of variations in shock-sensitivity. However differential shock-sensitivity could not account for example, for light-phase animals receiving more shocks on first trial, and less on second trial, in relation to other times of day.

II.7.2 Locomotor and social behaviour

The utility of social and aggressive behaviour as a comparative model for anxiety and other psychological disturbances, may seriously be questioned (a) because these parameters did not respond to benzodiazepine therapy (which should decrease emotionality) at the dosage employed, and (b) on the grounds of the near total dependence of these activities on olfactory stimuli, responsiveness to which may vary with time of day (Edwards, et al, I972). They are thus in no way applicable as a model for social effects in humans. They remain however, interesting in that one behaviour (aggression) appears to display the characteristics of an endogenously-determined rhythm,

while sociability does not. Clearly aggression should vary as a function of drive and neuromuscular capability (which it apparently does) though social cues / pheromones do play a vital role in the expression of both asocial and social behaviour. This is clearly shown by the fact that anosmic (sense of smell removed) mice will not fight or socially investigate (Edwards, et al, I972).

Similarly, variations in locomotor activity in mice should not be extrapolated as comparisons with human activity / rest patterns,particularly in the case of fatigue and the disturbance of sleep patterns in translocated subjects. The locomotor activity model however poses a useful objective method by which relationships with other behaviour patterns can be established.

II.8 Pharmacological manipulation

Many syptoms shown by air travellers show common links with people suffering from mental disturbances such as anxiety and insomnia (also associated with instability of rhythm patterns). As therapy for these complaints frequently involves the prescribing of benzodiazepine derivatives of both hypnotic and anxiolytic action, it follows that these drugs may prove useful in alleviating the symptoms of "jet lag". Indeed it is known that civil pilots frequently take these drugs (Preston & Bateman, I970), and a report has also been published, that disturbances of sleep patterns in shift workers may be prevented by benzodiazepine treatment (Ehrenstein, et al, I972).

The experiments described in this thesis have shown the long-acting benzodiazepines generally to provide the most satisfactory modifications to the passive avoidance response in mice. Generally-speaking, their effects are to:

(a) Impair the acquisition of PAR.

(b) Provide some measure of dose-dependent facilitation

(c) Prevent the retention deficits associated with phase shift, by employing a dosage which caused a behavioural change without causing neuromuscular impairment or behavioural depression.

A further important finding has been that of the "damping" effect of chlordiazepoxide treatment on the 24-hour rhythm for open-field behaviour, by inducing an increase in ambulation during the light phase, relative to other times. This portion of the LD cycle represents the period of lowest activity in the open field. The hypothesis has been that arousal is lowest, and fear greatest at this time. Thus benzodiazepine treatment in this case appears either to alleviate anxiety or to exert some other motivational effects by, for example, disinhibition. Clearly disinhibition could account for the failure to acquire the PAR, but not for heightened recall ability, while the drugs' anti-anxiety properties could indirectly facilitate recall. As recall is highest during the period of greatest anxiety, it seems therefore most likely that these drugs can facilitate memory processes by reducing anxiety, or possibly by reducing the heightened arousal as a result of exposure to shock. The learning of the avoidance task is not facilitated in the same way, possibly by the drug's known depressant action on the hippocampus (Schalleck & Thomas, I97I).

II.9 Pharmacological responsiveness at different times of day

The variation in the efficacy of many drugs at different times of day has clear implications for scientific research and clinical medicine, and there is now increasing awareness that allowances should be made for this phenomenon. For example, tolerance to pain may vary with time of day, and the symptoms displayed by various illnesses may also show marked daily fluctuations. For example recent investigations into rheumatoid arthritis (Harkness, et al, I98I)

have shown the symptoms of the disease to vary to such an extent that attendance at a morning or afternoon clinic could determine the kind of treatment given to a patient. Davies (I97I) has speculated as to the possibility of taking advantage of such a phenomenon, in that drugs with toxic side-effects could be administered at times of day when resistance to these side-effects was at its greatest.

The evidence presented in this thesis provides supportive evidence that the effectiveness of a drug will vary according to the time of administration, and may subsequently induce distortion of the 24-hour rhythm for the behavioural parameter in question (e.g. open-field and passive avoidance behaviour).

No clear and precise pattern emerges, as to when behavioural modification by benzodiazepine derivatives will be greatest, though in general, both long and short-acting derivatives appear to cause maximum effect in the early and mid light phase (chlordiazepoxide, clobazam, medazepam, diazepam). There are further complications in that some derivatives induce pronounced behavioural modifications (clobazam) while others cause changes only within narrow limits. The other classes of drugs tested appear not to differentiate on the same basis, tending to produce their maximum effects in the dark phase (with the exception of ethyl alcohol which did not substantially affect PAR at any time).

As much evidence exists for diurnal fluctuations in various aspects of brain chemistry, it follows that drugs which affect these biochemical components will themselves be subject to variations. It seems feasible to speculate that the specificity of neurotransmitter action of particular benzodiazepine derivatives will determine their action at different times of the day, to which other drugs (which possess different neurochemical actions) will act.

II.IO Neuro-mechanisms in passive avoidance

According to Carlton (I963; I966) and Carlton and Mankiewicz (I97I), central cholinergic systems are involved in the supression of unrewarded responses. Thus cholinergic transmission would be necessary for passive avoidance conditioning. Supportive evidence for this comes from the fact that treatment with muscariniolytic drugs such as atropine and scopolamine effectively impairs the response (Buresova, et al, I964; Meyers, et al, I964; Blozovski & Bachevalier,I974). Further, regional circadian variations have been demonstrated for acetylcholine receptors in the rat brain (Por & Bondy, I98I).

It has also been demonstrated that catecolaminergic mechanisms are involved in some stimulatory effects of chlordiazepoxide in laboratory animals (Vetulani & Sansone, I978). Also, Corrodi, et al (I97I) found evidence for decreased noradrenaline turnover following chlordiazepoxide and diazepam treatment, in the cerebral cortex and hippocampus. They also found a decrease in dopamine turnover in neurons ascending to the limbic forebrain. However it must be emphasized that these effects were observed after doses which induced sedation.

Wise, et al (I970) have put forward the suggestion that the anti-anxiety action of benzodiazepines depends on the inhibition of 5-Hydroxytryptamine turnover, whereas the depressant action seen at high doses depends on the depression of noradrenaline turnover.

It seems then that the benzodiazepines alter both the 5-HT and noradrenaline systems, though the mechanism by which the benzodiazepines suppress the 5-HT systems and the interrelationships with the noradrenaline system is unclear.

There is no firm evidence of 24-hour correlations obtained between simple learning tasks and catecholamine turnover, these have been characterised by higher frequency ultradian rhythms (Scheving, et al, I968). More recently it has been found that drugs which depleted forebrain noradrenaline levels affected neither acquisition nor retention of a passive avoidance task in rats (Mason & Fibiger,I979), though NA transmission has been implicated in the control of locomotor activity in mice (Vetulani & Sansone, I978).

However, a pronounced 24-hour variation in brain 5-HT levels has been well-reported (Scheving, et al, I968; Ancill, et al, I970) and the inverse relationship between rhythms in 5-HT and avoidance learning has been pointed out (Woolley & Van Hoeven, I965; Davies, et al, I974b), though a causal relationship remains to be firmly established. As previously mentioned in chapter 4, the therapeutic effects of benzodiazepines have also been ascribed to an influence by these drugs, on GABAergic mechanisms (Costa & Guidotti, I979),

II.II Possible mechanisms of photoperiodic time measurement

It seems that postulation of a neural, rather than an endocrine basis for the observed behavioural effects of light manipulation, is preferable, simply because of the rapidity of the effects. Lesions of the suprachiasmatic nucleus (SCN) have been reported to eliminate rhythmicity in plasma corticosterone levels in hamsters (Moore & Eichler, I972) and a variety of behaviours show degeneration of circadian rhythmicity following SCN lesions (Stephan & Zucker, I972). Neural control of rhythmicity (which may govern endocrine response) by the SCN, now appears not only to be important in the lower mammals (Zucker, et al, I975), but has been extended to both primates and man (Lydick, et al, I980).

Also recently, some investigators have postulated that the accomplishment of phase control of 24-hour rhythms may be via the retino-hypothalamic tract which originates in the retina, and monosynaptically innervates the SCN (Moore, I979).

Overall however, there now seems considerable supportive evidence for the central role of the SCN in controlling circadian rhythms in all mammals (reviewed by Rusak & Zucker, I979).

II.I2 Possible external influence: monthly and seasonal variation

Organisms in their natural environment possess simultaneously, a complex of biological rhythms of varying frequency. A number of small mammals such as rodents have been shown to possess at one and the same time, both lunar-day (Stutz, I974), seasonal and circannual cycles (Wurtman & Axelrod, I965; Pengelley & Asmundson, I97I; Peter, I98I; Adler, I980). While geophysical factors appear to control the former (Brown, I965), and daylength the latter (Reiter & Hester, I966), it is unclear as to the extent these cycles may pervade the laboratory and persist in experimental animals.

Some authors point out that laboratory animals have been and are influenced by subtle factors which penetrate laboratory "constant" conditions. Weak electrostatic fields, gamma radiation and geomagnetism penetrate easily through buildings and may influence timing mechanisms for biological clocks (Brown, I972, Brown & Chow, I973a). Recent evidence that mice are sensitive to, and can orientate in response to the earth's magnetic field has been presented (Mather & Baker, I98I). The presumed value of this ability is to confer "sense of direction" in nocturnal animals.

Little is known about whether seasonal cycles persist under laboratory conditions, but there is a suggestion that laboratory-maintained hamsters breed more readily in Spring than in

Winter, even under unchanging light and temperature conditions (Reiter, I973).

The possible persistence of lunar and seasonal variations in laboratory animals has not been accounted for in these experiments. At best one can minimise them by standardising environmental conditions and excluding external light and temperature cues.

II.I3 Relevance of the animal model

It has been frequently stated that phase shift-induced disturbances in animal studies, such as those employed in this thesis, should not be directly extrapolated as a model for "jet lag" effects in humans. It does however present an interesting paradigm for the objective study of internal desynchronization of 24-hour hour rhythms. However there are numerous obvious reasons for which comparisons should be treated with care. For one, the act of physical translocation has not taken place.

Any high-speed journey (especially Eastwards) over lines of longitude, will clearly be disturbing to the important daily routines of our lives. Gooddy (I97I) states....." It is not only the journey itself, but also the preparations over weeks or months.... .....and the new circumstances on arrival which contribute to our feelings of abnormality". Long periods involving confinement stress have also been found to lead to altertions of circadian rhythmicity in such factors as vigilance performance (Frazier, et al, I968).

Clearly disruption of routine in combination with internal desynchronization of bodily rhythms will combine to potentiate these subjective feelings of detachment, while sleep-patterns, motor co-ordination, mental efficiency and emotional stability may all be adversely affected. Clearly those most at risk from these symptoms are

those individuals delegated with some authority in terms of decision-making, such as ministers, diplomats, high-ranking military personnel and executives. Clear cases in point are the "shuttle diplomacy" missions of Henry Kissinger and Alexander Haig. Thus further investigations into dysrhythmic problems are vital, not only from the benefits which could accrue to travellers and the understanding of human chronometric activity, but more importantly, to circumvent problems associated with diplomatic activity at the highest level, and consequent effects on world peace.

Though not providing for the social / physical factors associated with human translocation, the animal model employed in this thesis offers a useful demonstration of some of the psychological effects of desynchronization, and is applicable in human terms in that:

(a) Susceptibility to therapeutic drugs will vary according to the time of day.

(b) Internal desynchronization in some psychological factors may be prevented by chronic drug therapy.

II.I4 Problems associated with drug therapy

The minor tranquillizers, like other psychoactive drugs appear to exert their effects in the brain centres associated with emotion and alertness, and when combined with alcohol can disturb perception and motor function. These effects are clearly undesirable in the context of the operation of sophisticated equipment, and the piloting of supersonic aircraft where reaction-time speed is at a premium. There is also evidence that large doses of benzodiazepines cause depression of pulse-rate, blood-pressure, respiration and drowsiness, as well as the risk of compulsive use and some measure of physical dependence. Withdrawl symptoms may include depression, agitation, insomnia and loss of apetite. Some individuals may further

display allergy-like reactions, while older individuals may suffer increased lethargy and slowness of reactions.

Clearly great care must be exercised in the use of these drugs, and individuals made aware of the possible side-effects. Discrimination should be exercised according to the nature of the work undertaken by a subject. For the pilot at the controls of an aircraft, where alertness and reaction speed are essential, the use of drugs could prove fatal. For the ordinary citizen however, the use of the minor tranquillizers, on balance, may prove of great benefit following long distance flight.

II.I5 Assessment of experimental methods

Ethologically-based studies such as those employed in this thesis, offer the advantage of minimising artefacts associated with laboratory conditions, and artificially-induced behaviour. The use of infra-red surveillance techniques, and other video-monitoring methods, has also been an advantage in that experimenter-interference has been eliminated. As a model for phase shift, these experimental methods offer the advantage of standardized conditions, whereas similar situations with humans are not realisable, due to the influence of a multiplicity of factors such as social / cultural variables and such complications as the effects of routine-disruption. For obvious reasons of practicality, human studies of fast transportation tend to involve a small number of subjects, whereas a large sample would be advisable to account for the substantial inter-individual differences in symptoms and adaptation periods (possibly correlated with personality differences; see Blake, I967b).

There is thus growing awareness that the study of circadian rhythms is of prime importance in the practice of air transport. The utility of animal studies such as these will, it is

hoped, provide information as to therapeutic possibilities, and to increase the awareness that psychological processes appear particularly vulnerable to sudden changes in the environment, both in animals, and in man.

SUGGESTIONS FOR FURTHER WORK.

(a) The experiments involving phase shift in this thesis, have not elucidated what critical period of light or dark time, in the 24 hours following shift, will predetermine a particular level of disruption. By employing phase-delays and advances of other than 6 hours, these critical periods could be correlated with their respective levels of disturbance.

(b) It could prove beneficial to extend the learning / retention model to other areas, by for example, the use of maze-learning tests.

(c) As the benzodiazepines employed in this study appear to alleviate the symptoms of phase shift, it could prove useful to examine the effect of all other benzodiazepine derivatives, and to differentiate them on the basis of their therapeutic (or otherwise) effects. The phase-shift test could even be used as a method for characterising the anxiolytic / psychomotor properties of other compounds. Further investigations could also be carried out to examine the action of other classes of clinically-important psychoactive drugs, such as those which possess a similar mode of action to the benzodiazepines.

(d) By the use of specific depletors, a detailed neuropharmacological examination could be conducted, to investigate the precise role of 5-HT and the catecholamines, in discrete regions of the brain, in relation to the 24-hour variation in acquisition and retention of the passive avoidance response. Brain levels of these neurotransmitters could be ascertained following phase shift, and their inter-relationship with benzodiazepine action could be examined at a histological level.

(e) Following on from the previous suggestion, it would be of interest to ascertain brain amine and nucleic acid turnover and metabolism, with respect to other behavioural parameters at different times of day, and further to examine the extent to which neurochemical and behavioural patterns are related, and to establish possible links between different behaviours on this basis.

(f) Amphetamine has been found to facilitate acquisition of avoidance tasks (e.g McGaugh & Petrinovitch, I965). Favourable effects have also been obtained by combining amphetamine with benzodiazepine derivatives (Sansone, I975; reviewed by Cooper, I979). Clearly this should be the subject of further research, particularly with respect to learning tasks.

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